

**Genetic engineering of the primary/secondary
metabolic interface in tobacco BY-2 cells**

Andrew Mark Hall-Ponselè

Michaelmas Term, 2013

D.Phil. thesis

University of Oxford

Department of Plant Sciences

New College

Supervisors: Dr Lee J. Sweetlove and Professor R. George Ratcliffe

Abstract

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Andrew Mark Hall-Ponselè, New College

Submitted for the degree of D.Phil., Michaelmas Term, 2013

The supply of precursors from primary metabolism is often overlooked when engineering secondary metabolism for increased product yields. This is because precursor supply may be assumed to be non-limiting, and it is considered difficult to engineer primary metabolism, because control of carbon flow (flux) is generally distributed among most enzymes of the pathway.

The aim of this thesis was to increase the production of sterols, part of the isoprenoid class of secondary metabolites, in tobacco (*Nicotiana tabacum*) Bright Yellow 2 (BY-2) cell cultures. This was achieved by genetically engineering increased activity of mitochondrial citrate synthase, an enzyme of the tricarboxylic acid (TCA) cycle that is involved in the provision of cytosolic acetyl coenzyme A, the primary metabolite precursor to sterols. Metabolic flux analysis revealed that citrate synthase exerts significant control over cyclic TCA cycle flux in BY-2 cells and suggested that increasing the activity of downstream enzymes within secondary metabolism could lead to a further redirection of TCA-cycle-derived precursors into sterol biosynthesis. Attempts were made to achieve this by genetically engineering increased activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), a key enzyme of secondary metabolism involved in sterol biosynthesis. Consistent with previous research, transgenic lines had increased sterol levels. However, the high sterol phenotype was unstable, and attempts to co-express HMGR and citrate synthase genes were unsuccessful.

The thesis demonstrates that increasing the provision of precursors to secondary metabolites can result in increased yields of those secondary metabolites but suggests that in most cases the activity of enzymes within secondary metabolism has a greater effect on those yields. It also reveals that single enzymes can exert significant control of flux within primary metabolism, although the control exerted by specific enzymes probably changes with the demands placed on metabolism.

Word count = Approximately 47,000

Acknowledgments

I thank Dr Lee Sweetlove and Professor George Ratcliffe for devising the project and giving it direction. I am indebted to them for the efforts they put into assessing my work over the years and for transforming me from a student into a scientist. I have an overwhelming appreciation for their knowledge, foresight and precision. It has been a privilege to be supervised by two such leading experts in their fields.

I acknowledge the many excellent scientists in the biochemistry lab with whom I have had the honour of working. In particular I would like to thank Dr Shyam Masakapalli, for teaching me nearly everything I know about the implementation of metabolic flux analysis, and Dr Nick Kruger, for refining my knowledge of that discipline. I also thank Mr Pedro Bota for his help in the laboratory, especially with the GC-MS, and Dr Katherine Beard, Mr Constantine Garagounis, Mrs Aliah Hawari, Miss Gemma Hayton, Dr Annette Kugler and Miss Clare Woods for their moral support in and around the department.

I also thank Dr Genny Enfissi and Dr Monika Kalde for genetic constructs and Dr Katja Grauman for the wild-type BY-2 cells. I recognise the BBSRC and New College for their financial assistance.

Most importantly, I thank my wife, Elisa, for her love, patience and encouragement. Her support over these past few years has been vital. By extension, I would like also to thank my family and friends.

Abbreviations

Acetyl-CoA	Acetyl coenzyme A
ACL	ATP-citrate lyase
AGE	Agarose gel electrophoresis
ANOVA	Analysis of variance
APAD	Acetylpyridine adenine dinucleotide
ATP	Adenosine triphosphate
BY-2	Bright Yellow 2
CaMV	Cauliflower mosaic virus
CCE	Carbon conversion efficiency
CCF	Cell culture filtrate
cDNA	Complementary DNA
CS	Citrate synthase
DMAPP	Dimethylallyl diphosphate
DTC	Dicarboxylate-tricarboxylate carrier
DTT	Dithiothreitol
DW	Dry weight
DXP	1-Deoxy-D-xylulose-5-phosphate
DXS	1-Deoxy-D-xylulose-5-phosphate synthase
EDTA	Ethylenediaminetetraacetic acid
FADH ₂	Reduced flavin adenine dinucleotide
FBA	Flux balance analysis
FPP	Farnesyl diphosphate
FW	Fresh weight
G3-P	Glyceraldehyde 3-phosphate
GABA	γ-amino-butyrate
GC	Gas chromatography
GC-MS	Gas chromatography coupled to mass spectrometry
GGPP	Geranylgeranyl diphosphate
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
HMGR	3-Hydroxy-3-methylglutaryl coenzyme A reductase
IDH	Isocitrate dehydrogenase
IEC	Ion exchange chromatography

IPP	Isopentenyl diphosphate
MANOVA	Multivariate analysis of variance
MCS	Monte Carlo simulations
MEOX	Methoxyamine
MEP	2- <i>C</i> -Methyl-D-erythritol 4-phosphate
MFA	Metabolic flux analysis
MS	Mass Spectrometer
MSTFA	<i>N</i> -Methyltrimethylsilyltrifluoroacetamide
m/z	Mass-to-charge ratio
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NDA	Nonadecanoic acid
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEP	Phospho <i>enol</i> pyruvate
PPM	Parts per million
PPP	Pentose phosphate pathway
QTL	Quantitative trait locus
SEM	Standard error of the mean
TBDMS	<i>t</i> -Butyldimethylsilyl
TCA	Tricarboxylic acid
TLC	Thin layer chromatography
TMS	Trimethylsilyl
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
WT	Wild-type

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Chapter 1. Introduction

1.1. Introduction

This introductory chapter discusses the rationale for engineering improved yields of secondary plant metabolites. It covers traditional approaches to manipulating secondary metabolism in plants, focusing on attempts to increase yields of isoprenoids, particularly the carotenoids. These early attempts at metabolic engineering met with only limited success, largely due to a lack of consideration of the effects on the wider metabolic network. More recently, considerations of the metabolic network as a whole have become more prominent with the emergence of systems biology. This is particularly so in bacterial metabolic engineering, where synthetic biology has also facilitated considerable progress. The chapter goes on to describe how sterol biosynthesis provides a viable example of a plant secondary metabolic pathway that may be manipulated to produce large product yields. Finally, the objectives and approaches of the thesis are summarised.

1.2. Genetic engineering of secondary metabolism

1.2.1. The rationale for engineering plant secondary metabolism

Secondary metabolites are usually considered to be taxonomically-restricted compounds that are inessential for a cell or organism's survival but are involved in interactions with its ecological niche (Verpoorte, 2000). They make up nearly all of the up to 200,000 distinct chemical species that are estimated to exist in the plant kingdom (Fiehn, 2002). For the sake of this thesis, secondary metabolites are considered more generally to be the products of the

many branching pathways that emerge from the highly connected catabolic and anabolic core of primary metabolism. These include those inessential compounds traditionally considered to be secondary metabolites but also include many biosynthetically related metabolites necessary for cell survival and reproduction, such as ubiquinone and the sterols.

Plant secondary metabolites include compounds involved in photosynthesis, defence and attraction of pollinators and seed dispersers. Many of these have nutritional or pharmaceutical qualities and are a major source of revenue for the pharmaceutical, food and cosmetics industries. They are frequently chemically complex, making chemical synthesis expensive or infeasible (Avery et al., 1992; Danishefsky et al., 1996; Nicolaou et al., 1997), and they are often naturally synthesised in insufficient quantities to meet demand by humans. Increasing yields of specific secondary metabolites in plants could result in substantial medical, economic and environmental benefits by increasing supplies of medicines, increasing land-use efficiency and creating highly nutritious feedstock.

Conventional breeding is capable of increasing the secondary metabolite yields of plants. For instance, natural mutations in β -*Lcy*, encoding lycopene β -cyclase, and ϵ -*Lcy*, encoding lycopene ϵ -cyclase, have been demonstrated to increase the content of β -carotene, the carotenoid precursor to vitamin A, in tomato (*Solanum lycopersicum*) fruit (Ronen et al., 2000) and maize (*Zea mays*) endosperm (Harjes et al., 2008), respectively. However, conventional breeding is extremely time-consuming. It also requires the ability to accurately map each quantitative trait locus (QTL), the use of small populations to select for these QTLs may result in inadvertent selection of other undesirable agronomic traits such as loss of vigour, and the process must be iterated in order to transfer the QTLs to locally adapted varieties or to increase the yields of additional metabolites (Naqvi et al., 2009). Also, a lack of genetic diversity within a species limits the extent of change that is possible. By comparison, genetic

engineering allows the production of compounds from across the natural spectrum, as well as non-natural compounds (Zhang et al., 2008; Zhang et al., 2010), at higher than natural levels.

1.2.2. Traditional approaches to metabolic engineering in plants

Traditional approaches to engineering secondary metabolism in plants generally involve the over-expression of genes encoding enzymes thought to exert significant control over flux within the specific branch of metabolism. Complementary techniques developed later seek to reduce flux into competing pathways, increase sequestering of the pathway product or use regulatory genes to alter the expression of several genes simultaneously (Figure 1.1).

Little consideration was apparently given to the provision of precursors to the secondary metabolic pathways in early experiments. This meant that, when successful, the approach could increase flux towards the desired branch of metabolism and deplete the pool of intermediates available to necessary competing upstream reactions. Thus, these early attempts at metabolic engineering frequently resulted in undesirable and unforeseen pleiotropic effects (Fray et al., 1995; Shewmaker et al., 1999; Besumbes et al., 2004; Fraser et al., 2007; Kovacs et al., 2007). Where such effects did not occur, product yields tended to be disappointingly low (Misawa et al., 1993; Misawa et al., 1994; Römer et al., 2000) due to the lack of understanding about metabolic pathway regulation, particularly with respect to differential regulation in different tissues and developmental states.

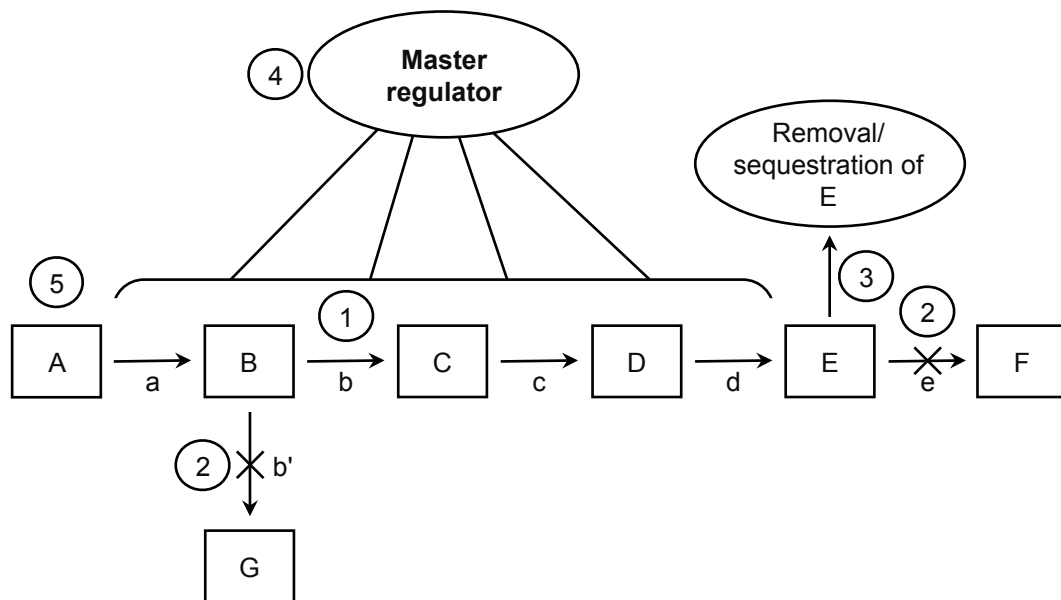


Figure 1.1. Different approaches to engineering greater yields of a hypothetical secondary metabolite (E).

A primary metabolite (A) is converted by various enzymes (lower-case letters) into the desired product, E. To increase the yield of E the activity of single biosynthetic enzymes may be increased (1), activity of enzymes in competing pathways may be reduced (2), the desired metabolite may be removed from the metabolic pool by either storing subcellularly or excretion (3), the expression of a relevant transcription factor may be altered (4), or the supply of the pathway precursor from primary metabolism may be increased (5). (Figure adapted from Oksman-Caldente and Inzé (2004).)

Overview of isoprenoid biosynthesis

This review primarily considers the metabolic engineering of isoprenoids. Isoprenoids are one of the four major biosynthetically distinct groups of plant secondary metabolites (Verpoorte, 2000; Croteau et al., 2002) and are the most structurally and functionally diverse (Rodríguez-Concepción and Boronat, 2002). They derive from the five-carbon isomers isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Ruzicka, 1953; Chappell, 1995a). They include photosynthetic pigments, hormones and structural components of membranes.

Plant isoprenoids are products of two distinct pathways, both of which produce IPP and DMAPP (Figure 1.2). IPP and DMAPP precursors to plastidial isoprenoids are synthesised in the plastid via a 2-C-methyl-D-erythritol 4-phosphate (MEP) intermediate (Lichtenthaler et al., 1997; Hsieh et al., 2008), whereas non-plastidial isoprenoids derive from precursors synthesised via a mevalonate intermediate using a pathway split between the cytosol, endoplasmic reticulum and peroxisome (Denbow et al., 1996; Leivar et al., 2005; Nagegowda et al., 2005; Carrie et al., 2007; Merret et al., 2007; Sapir-Mir et al., 2008; Simkin et al., 2011). A limited degree of crossover of intermediates can occur between the two pathways *in vivo* (Bick and Lange, 2003; Hemmerlin et al., 2003; Laule et al., 2003). Successive additions of IPP to DMAPP create isoprenoid precursor compounds of different lengths and cause the wide natural diversity of the metabolite group.

Carotenoids branch from the MEP pathway when phytoene is formed by the condensation of two molecules of the 20-carbon geranylgeranyl diphosphate (GGPP) (Cunningham and Gantt, 1998) (Figure 1.3). Carotenoids confer various health benefits to humans (Rao and Rao, 2007). Most notably α -carotene, β -carotene and β -cryptoxanthin are converted by the body into vitamin A (Rao and Rao, 2007). Severe vitamin A deficiency causes night blindness and immunodeficiency in an estimated 15 million pregnant women and preschool-aged children

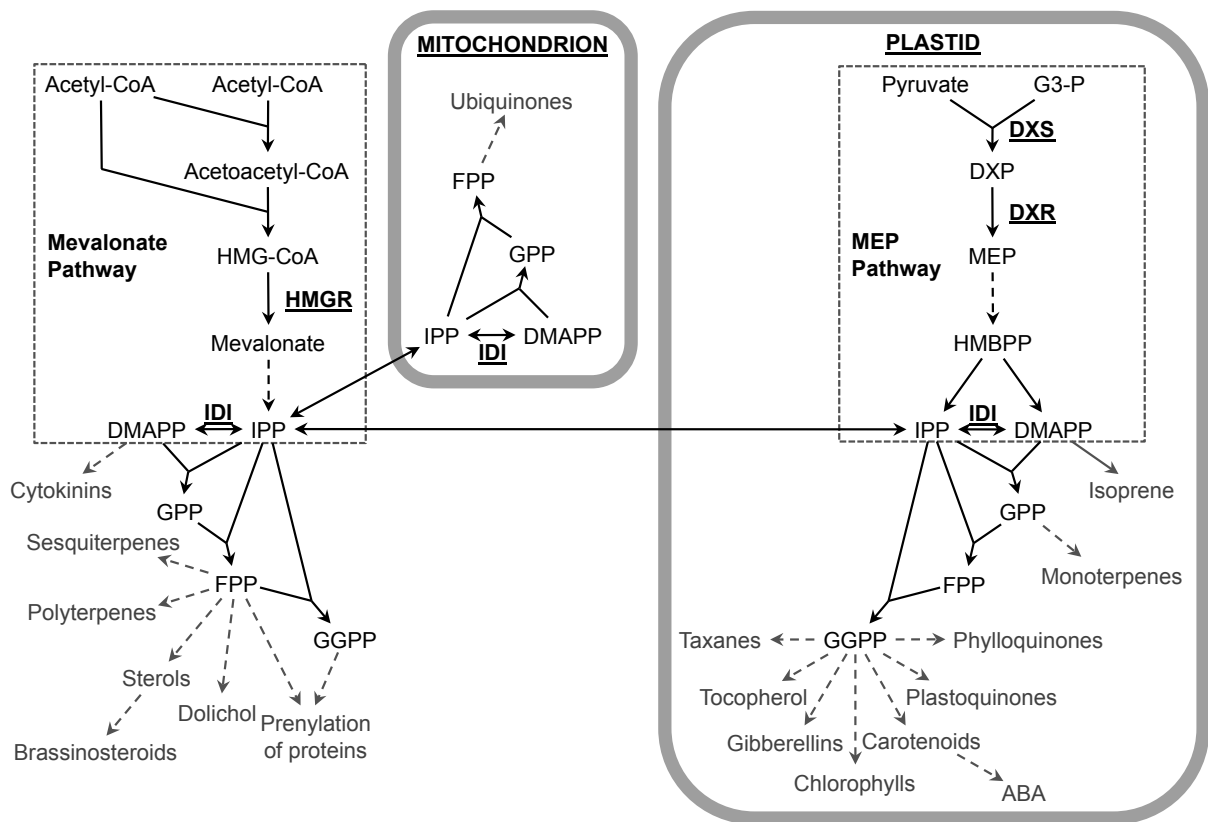


Figure 1.2. Schematic representation of isoprenoid biosynthesis in plant cells.

Non-plastidial isoprenoids are synthesised from IPP and DMAPP created via the mevalonate pathway (Bach and Lichtenthaler, 1983; McGarvey and Croteau, 1995; Chappell, 1995b). Plastidial isoprenoids are synthesised from IPP and DMAPP created via the MEP pathway (Eisenreich et al., 1996; Lichtenthaler et al., 1997). Not shown: Limited exchange of GPP and FPP in addition to IPP is thought to occur between the plastid and the cytosol (Bick and Lange, 2003; Hemmerlin et al., 2003; Laule et al., 2003). Enzymes mentioned in the text are highlighted.

Abbreviations: **ABA**, abscisic acid; **DMAPP**, dimethylallyl diphosphate; **DXP**, 1-deoxy-D-xylulose-5-phosphate; **DXR**, DXP reductase; **DXS**, DXP synthase; **FPP**, farnesyl diphosphate; **G3-P**, glyceraldehyde 3-phosphate; **GGPP**, geranylgeranyl diphosphate; **GPP**, geranyl diphosphate; **HMBPP**, hydroxymethylbutenyl 4-diphosphate; **HMG-CoA**, 3-Hydroxy-3-methylglutaryl-CoA, **HMGR**, HMG-CoA reductase; **IDI**, isopentenyl diphosphate isomerase; **IPP**, isopentenyl diphosphate; **MEP**, 2-C-methyl-D-erythritol 4-phosphate.

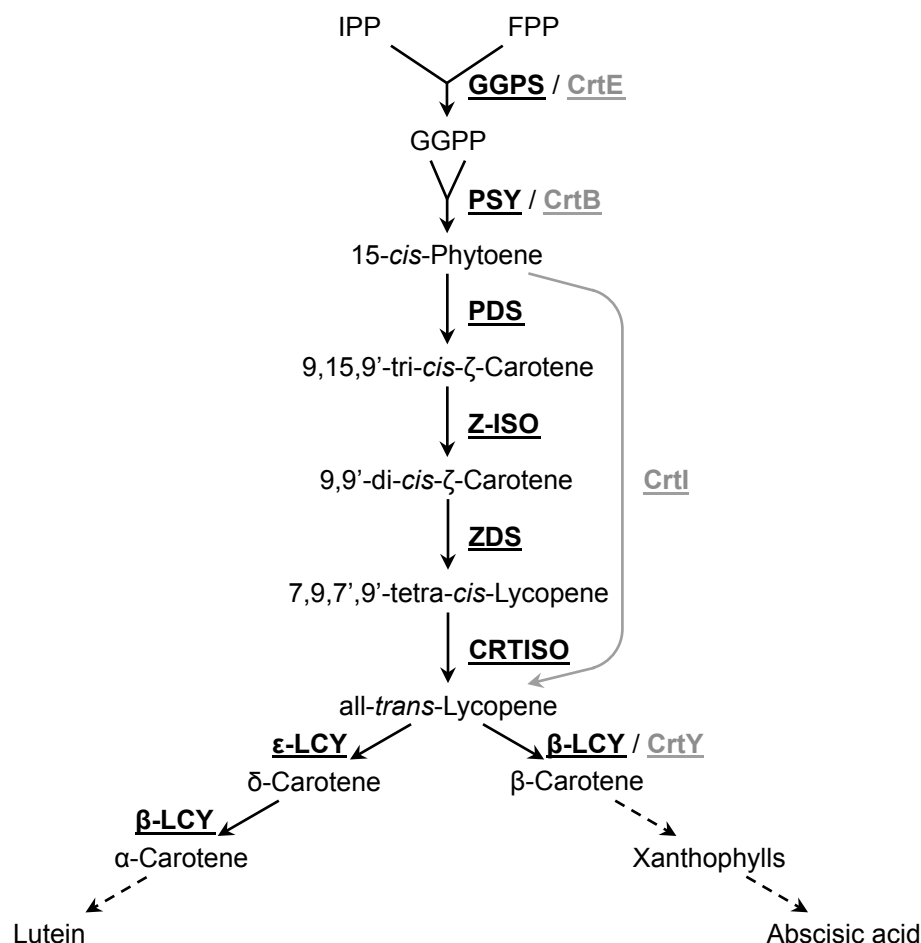


Figure 1.3. Schematic representation of carotenoid biosynthesis.

Carotenoids are formed following the condensation of two molecules of geranylgeranyl diphosphate (GGPP) by phytoene synthase (PSY). Enzymes at each step are underlined. Those in black are plant enzymes, while those in grey (CrtE, CrtB, CrtI and CrtY) are bacterial.

Abbreviations: **β-LCY/CrtY**, lycopene β-cyclase; **CrtI**, bacterial phytoene desaturase; **CRTISO**, carotenoid isomerase; **CrtY**, bacterial lycopene β-cyclase; **ε-LCY**, lycopene ε-cyclase; **GGPP**, geranylgeranyl diphosphate; **GGPS/CrtE**, GGPP synthase; **PDS**, plant phytoene desaturase; **PSY/CrtB**, phytoene synthase; **ZDS**, ζ-carotene desaturase; **Z-ISO**, ζ-carotene isomerase.

globally and is a leading cause of preventable infant and pregnancy-related mortality in less developed countries (World Health Organization, 2009). Attempts have been made to increase β -carotene yields in edible plant tissues in efforts to achieve a daily intake of pro-vitamin A of 6 mg (Rosati et al., 2000) in regions where nutrient-poor diets currently persist.

Targeting increased activity of single biosynthetic enzymes

Initial experiments into altering carotenoid yields involved targeting single biosynthetic enzymes. These were often expressed from foreign genes, as co-suppression frequently occurred when transforming plants with an endogenous gene (Fray and Grierson, 1993; Fray et al., 1995; Fraser et al., 2007). Tobacco (*Nicotiana tabacum*) plants constitutively expressing a plastid-targeted *PHYTOENE DESATURASE* (*crtI*) from *Erwinia uredovora* produced a higher proportion of leaf β -carotene, but this was at the expense of phytoene and ζ -carotene (Misawa et al., 1993; Misawa et al., 1994). Attempts to increase lycopene content in tomato fruit using *crtI* unexpectedly increased transcription of the endogenous *PHYTOENE DESATURASE* (*Pds*) and ζ -*CAROTENE DESATURASE* and caused yields of lycopene derivatives (most notably β -carotene) to increase (Römer et al., 2000), although lycopene and overall carotenoid levels were reduced. This reflects a reduction in phytoene synthase transcription, probably due to accumulating downstream carotenoids causing feedback transcriptional inhibition (Römer et al., 2000), and is an indication of the difficulty of accurately predicting the effects of genetic manipulations to metabolism.

Despite failure to increase carotenoid levels in tomato fruit through constitutive *crtI* expression, increases were achieved through the over-expression of *LYCOPENE BETA-CYCLASE* (β -*Lcy*) controlled by the endogenous *Pds* promoter (Rosati et al., 2000), which is up-regulated in fruit during ripening (Giuliano et al., 1993; Corona et al., 1996). This overcame the natural ripening-associated reductions in β -LCY activity (Pecker et al., 1996;

Ronen et al., 1999) resulting in the accumulation of β -carotene and a doubling of total carotenoid content in ripe fruit. These results demonstrate that increasing flux into downstream products can cause total yields of a metabolite group to increase.

Other work showed a role for *PHYTOENE SYNTHASE* (*Psy-1*) in regulating carotenoid levels in tomato. Constitutive over-expression increased carotenoid levels in carotenoid-deficient mutant (Fray and Grierson, 1993) and wild-type backgrounds (Fray et al., 1995; Fraser et al., 2007) and was associated with the accumulation of carotenoids in novel tissues. The increases were due to feedback and feed-forward regulation increasing activity of 1-deoxy-D-xylulose-5-phosphate (DXP) synthase (DXS) and downstream enzymes, respectively (Fraser et al., 2007).

Increasing flux into carotenoids through manipulations to phytoene synthase activity often led to the depletion of GGPP and decreases in other GGPP-derived isoprenoids (Fray et al., 1995; Shewmaker et al., 1999; Fraser et al., 2007), and so caused undesirable phenotypes. For instance, decreased gibberellin levels resulted in dwarfism in tomato plants constitutively over-expressing *Psy-1* (Fray et al., 1995; Fraser et al., 2007). Depletion of GGPP is less detrimental when tissue- and development-specific transcription limits the depletion to metabolic sink organs after growth. Thus β -carotene levels have been increased by tissue-specific over-expression of phytoene synthase genes in tomato fruit (from 330 to 825 $\mu\text{g (g DW)}^{-1}$) (Fraser et al., 2002) and *Brassica napus* seeds (from 3 to 949 $\mu\text{g (g FW)}^{-1}$) (Shewmaker et al., 1999) without serious consequences for the rest of the plant.

Improvements in vector design have allowed for co-transformation of plants with several genes, allowing the introduction of entire heterologous biosynthetic pathways into novel tissues and species. A pathway from GGPP to β -carotene has been introduced into rice

endosperm (Ye et al., 2000), potato tubers (Diretto et al., 2007) and maize (*Zea mays*) kernels (Naqvi et al., 2009) by co-transformation with *Psy-1*, *crtI*, and *β -Lcy*. β -carotene yields in co-transformed rice were improved by approximately 16 times by switching from daffodil (*Narcissus pseudonarcissus*) to maize *Psy-1* (Paine et al., 2005). A high GGPP to phytoene ratio suggested that a more efficient promoter or enzyme could give further yield increases. However, attempts to move the biosynthetic operon into commercial rice strains have resulted in disappointing yields of β -carotene (Datta et al., 2003; Hoa et al., 2003; Baisakh et al., 2006; Datta et al., 2007). This could be a result of a more limited availability of GGPP in these strains.

Targeted decreases of flux into competing pathways

Increased yields from one branch of a pathway can be achieved through limiting the conversion of a desired metabolite (or its precursor) into an undesired metabolite. For instance, antisense suppression of *β -Lcy* in tomato fruit reduced flux from lycopene to β -carotene, causing a doubling of lycopene content (Rosati et al., 2000). Similarly, the proportion of β -carotene in the carotenoid yield of potato (*Solanum tuberosum*) tubers was increased through antisense suppression of *LYCOPENE EPSILON-CYCLASE* (*ϵ -Lcy*) (Diretto et al., 2006). This reduced flux from lycopene into the α -carotene branch of carotenoid metabolism, causing a redirection of flux into the β -carotene branch. Total carotenoid yields increased slightly (up to 100%) in both examples. This could be due to pathway sensitivity to the altered metabolite or enzyme levels.

Product sequestration

Other successes at increasing carotenoid yields have involved targeting the development and structure of the plastid, in order to sequester the product and shift the thermodynamic equilibrium towards product formation. A doubling of carotenoid levels in tomato fruit was

achieved by the expression of a gene encoding a pepper (*Capsicum annuum*) fibrillin (Simkin et al., 2007), a chromoplast structural protein associated with carotenoid accumulation (Deruère et al., 1994a; Deruère et al., 1994b; Vishnevetsky et al., 1999). Increased plastid number and volume also seems to cause increased carotenoid yields (Cookson et al., 2003; Enfissi et al., 2010; Liu et al., 2004; Powell et al., 2012), although this may be primarily due to corresponding increases in the numbers of plastidial enzymes involved in carotenoid biosynthesis rather than an increased ability to sequester carotenoids. Carotenoid-binding proteins from cyanobacteria or algae might also be used to improve carotenoid yields in plants (Vishnevetsky et al., 1999). Similarly RNA interference has demonstrated a positive correlation between triacylglycerol and carotenoid levels in the unicellular alga *Dunaliella bardawil* (Rabbani et al., 1998). It is possible that triacylglycerols sequester carotenoids and that increasing triacylglycerol synthesis in higher plants would similarly affect carotenoid yields.

Manipulation of regulatory genes

Phenotypes caused by altered levels of transcription factors indirectly related to secondary metabolism can include altered levels of secondary metabolites. For instance, transcriptional inhibition of and mutations in *DE-ETIOLATED1* (*DET1*; a regulatory gene involved in the repression of light-induced genes) caused dwarfism and the accumulation of carotenoids and flavonoids (Yen et al., 1997; Mustilli et al., 1999; Levin et al., 2003; Davuluri et al., 2004; Bino et al., 2005). These major phenotypic changes were caused by the up-regulation of light-responsive pathways, including photosynthesis and photorespiration, and a subsequent increase in the rate of primary metabolism (Enfissi et al., 2010). Fruit-specific transcriptional inhibition of *DET1* in tomato overcame the dwarfism apparent in constitutive silencing (Davuluri et al., 2005; Enfissi et al., 2010) and resulted in an increase in total carotenoids to six times the control values (to 4.2 mg (g DW)⁻¹), including an eight-fold increase in β -carotene content (to 1.5 mg (g DW)⁻¹) (Enfissi et al., 2010). These increases reflect an increase in *PHYTOENE SYNTHASE 2* and *GGPP SYNTHASE* transcripts and may have been greater if

not for apparent feedback inhibition of *Dxs*. Flavonoids and phenylpropanoids were also increased, indicating the broadness of the response.

Over-expression of *Or*, which is implicated in differentiation of non-coloured plastids into chromoplasts, in cauliflower (*Brassica oleracea* var. *botrytis*) (Lu et al., 2006) and potato tuber (Lopez et al., 2008) has also caused novel accumulation of carotenoids. β -carotene yields in potato tubers were less than half of those achieved through the expression of a heterologous biosynthetic pathway (Diretto et al., 2007), but they were achieved through over-expression of a single gene (Lopez et al., 2008).

Another approach, that can be similar in effect to altering transcription factor expression, is to manipulate chromatin structure. For instance, a mutation in the histone methyltransferase gene *CAROTENOID CHLOROPLAST REGULATORY1* altered carotenoid composition in Arabidopsis (*Arabidopsis thaliana*) by epigenetically reducing expression of *CAROTENOID ISOMERASE* (Cazzonelli et al., 2009).

1.2.3. The relevance of primary metabolism for engineering of secondary metabolism

Evidence for the impact of the provision of primary metabolites on the productivity of secondary metabolism

Little research has been done explicitly into interactions between primary metabolism and secondary metabolism in plants in a metabolic engineering context, although this is increasingly being considered (Aharoni and Galili, 2010). Very high flux of acetyl coenzyme A (acetyl-CoA) into a heterologous polyhydroxybutyrate pathway in Arabidopsis appears to

deplete plastidial acetyl-CoA from competing pathways (Bohmert et al., 2000). Other examples cited previously have described the deleterious effects of depleting precursors for one or more groups of metabolites by increasing flux into a related pathway (Fray et al., 1995; Shewmaker et al., 1999; Bohmert et al., 2000; Besumbes et al., 2004; Fraser et al., 2007; Kovacs et al., 2007) and the limitations imposed by a finite pool of precursors on the yield increase achieved by targeted pathways (Misawa et al., 1993; Misawa et al., 1994; Fray et al., 1995; Römer et al., 2000; Rosati et al., 2000). All of these examples are suggestive of some degree of limitation in precursor availability.

Conversely, some perturbations to plant primary metabolism have been demonstrated to affect secondary metabolism. Reductions in ATP-citrate lyase activity in *Arabidopsis* caused dwarfism and accumulation of anthocyanins (Fatland et al., 2005), and down-regulation of transketolase in tobacco also resulted in dwarfism and decreases in carotenoid and tocopherol levels (Henkes et al., 2001). However, it is difficult to demonstrate a direct causal link between reduced primary metabolic flux and altered secondary metabolic flux in these examples; the pleiotropic effects could instead be stress responses operating through less obvious signalling cascades.

A more explicit example of the inter-relatedness of primary and secondary metabolism was demonstrated by the effects of reduced enolase activity in tobacco plants (Voll et al., 2009). In this example, a resultant reduction in phosphoenolpyruvate levels caused a decrease in flux through the shikimate pathway and, consequently, decreased contents of phenylalanine and phenylalanine-derived secondary metabolites. Correlations have also been shown between the availability of nitrogen-rich primary metabolites and the yield of nitrogen-rich secondary metabolites (Fritz et al., 2006) and between the ratios of carbon-rich to nitrogen-rich primary metabolites and carbon-rich to nitrogen-rich secondary metabolites (Matt et al., 2002),

suggesting that secondary metabolism may provide a route for excess precursors from primary metabolism.

The multi-faceted effects of transcription factors provide further evidence of the interdependence of primary metabolism and secondary metabolism. For instance, transcription factors that activate expression of genes involved in the biosynthesis of the aliphatic and indole glucosinolates also activated expression of genes for biosynthesis of methionine and tryptophan, their respective primary metabolite precursors (Malitsky et al., 2008).

Application of systems biology to metabolic engineering

Metabolic engineering has recently begun to be viewed more holistically. This has been facilitated by the emergence of systems biology. High-throughput mass spectrometry-based methods have yielded metabolomic information, while the increased availability of genomic information has facilitated greater predictive power from genome-scale metabolic models. This is particularly apparent in the literature on microbial metabolic engineering.

The provision of primary metabolite precursors has been manipulated to increase production of secondary metabolites in bacteria. *Escherichia coli* synthesises isoprenoids via the MEP pathway. Farmer and Liao (2001) extended this pathway to lycopene biosynthesis in *E. coli*. They then demonstrated the importance of the ratio of pyruvate to glyceraldehyde 3-phosphate (G3-P) available to the MEP pathway by making genetic perturbations to influence the flux between these two primary metabolites. They found that over-expressing phosphoenolpyruvate (PEP) synthase or PEP carboxykinase genes, or inactivating pyruvate kinase genes, increased the proportions of G3-P to pyruvate and resulted in increased yields of lycopene. Overexpression of *PEP CARBOXYLASE* had the opposite effects. Such precursor balancing may be relevant to modifications of the MEP pathway in plants and may have

relevance for other pathways of secondary metabolism, particularly when expressing a novel pathway in a host that does not possess the means of regulating it.

Metabolic engineering of *E. coli* for lycopene biosynthesis has also been informed by flux balance analysis (FBA) (Alper et al., 2005), a computational approach to modelling metabolic fluxes. FBA predicted that knocking out *GLUTAMATE DEHYDROGENASE* (*gdhA*) would increase the availability of NADPH, while knockout of *PYRUVATE DEHYDROGENASE* (*aceE*) was predicted to increase pyruvate availability for the MEP pathway. Knockout of *FORMATE DEHYDROGENASE* (*fdhF*) was predicted to increase lycopene yields in *aceE* knockout lines by reducing a predicted redirection of flux through formate when *aceE* is knocked out. Titres of lycopene in lines already overexpressing MEP pathway genes were increased 40% to 6.6 mg (g DW)⁻¹ by knockout of all three candidates, although growth rate was reduced by nearly 50%.

Later experiments were undertaken based on FBA predictions of gene targets for overexpression in order to gain increased lycopene titres in *E. coli* (Choi et al., 2010). Manipulating enzymes in primary metabolism was again suggested in addition to enzymes of the MEP and carotenoid biosynthesis pathways. In particular, availability of G3-P was estimated to be critical. Knockout of *gmpB* (one of two genes encoding phosphoglycerate mutase) and *gdhA* successfully increased lycopene production. Tricarboxylic acid (TCA) cycle fluxes were also targeted in order to increase the availability of ATP and reducing power for synthesis of IPP and DMAPP. Overexpression of *MALATE DEHYDROGENASE* (*mdh*) and *ISOCITRATE DEHYDROGENASE* (*icdA*) each increased lycopene yields. The most productive line, combining *gmpB* and *gdhA* knockout with *mdh* and *IPP ISOMERASE* (*idi*) overexpression in a line already overexpressing MEP pathway genes, had lycopene yields of 12.3 mg (g DW)⁻¹.

Other research not based on FBA has also highlighted the importance of cofactor availability for carotenoid biosynthesis in transgenic *E. coli* (Zhao et al., 2013). Zhao et al. expressed the *Pantoea agglomerans* β -carotene biosynthesis pathway in *E. coli* and conceptually divided it into five modules: β -carotene synthesis, the MEP pathway, ATP synthesis, the TCA cycle and the pentose phosphate pathway (PPP). Alteration of the promoter strength for genes in these modules led to the production of 30 mg (g DW)⁻¹ β -carotene, the highest published yields by *E. coli*. It was noted in this work that the highest expression of the β -carotene biosynthesis operon was only achievable when the supply of IPP and DMAPP had already been increased by modulating expression of *dxs* and *idi* in the MEP pathway module. This was due to the potential for overwhelming competition by β -carotene biosynthesis for its substrates. Several up-regulatory combinations of the TCA cycle were attempted. The best β -carotene yields resulted from up-regulation of α -KETOGLUTARATE DEHYDROGENASE (*sucAB*), CITRATE SYNTHASE and SUCCINATE DEHYDROGENASE (*sdhABCD*). *sucAB* and *sdhABCD* had synergistic effects and gave the greatest increases in β -carotene yield. Modulating TRANSKETOLASE I (*tktA*) and TRANSALDOLASE B (*talB*) of the PPP also increased β -carotene yield. Modulating genes of the TCA cycle had a greater effect than ATP synthesis genes or PPP genes on β -carotene yield. Combining manipulations of the TCA cycle and the PPP (*sucAB* and *talB*) resulted in further β -carotene yield increases, although combining *sucAB* modulation with *tktA* modulation led to a decrease in β -carotene yield, as did combination of *sucAB* with ATP synthesis genes. The lack of synergism between TCA cycle modulation and ATP synthesis modulation suggested that NADPH supply was more critical than ATP supply, although ATP is supplied, in part, by the TCA cycle. The results also indicate the difficulty in predicting which aspects of metabolism are most relevant to specific pathways *a priori*.

Screening of a yeast (*Saccharomyces cerevisiae*) gene deletion collection for deletions that improved carotenoid titres in yeast also highlighted novel gene targets, as well as transcription factors

regulating mevalonate pathway genes (Özaydın et al., 2013). Deletion of genes involved in lipid synthesis (*lacI*) and amino acid synthesis (*ser33*) improved carotenoid yields, possibly due to the antioxidant qualities of carotenoids being favoured in cells with a disrupted redox balance. It was noted that the same deletions did not result in increased titres of an alternative isoprenoid, bisabolene, in yeast expressing a bisabolene biosynthesis pathway instead of the carotenoid biosynthesis, which supports the idea that the manipulations did not cause a general redirection of flux through the MEP pathway.

1.2.4. Further considerations

Lessons from microbial synthetic biology

Other work on improving isoprenoid titres in *E. coli* has highlighted the advances that can be made using synthetic biology. Martin et al. (2003) expressed a codon-optimised mevalonate pathway in *E. coli* to bypass control elements associated with the endogenous MEP pathway. They organised this into two operons: the production of mevalonate from acetyl-CoA, and the production of farnesyl diphosphate (FPP) from mevalonate (Figure 1.4). This was done so that mevalonate could be assayed to determine the activity of operon one (Martin et al., 2003) or could be added to the medium to assess the activity of operon two (Pfleger et al., 2007).

It was discovered that the initial *E. coli* strain accumulated toxic prenyl diphosphates (IPP, DMAPP and FPP), which inhibited growth (Martin et al., 2003). This toxicity could be alleviated by co-expressing *AMORPHADIENE SYNTHASE*, which encodes the enzyme catalysing the first committed step in the biosynthesis of the anti-malarial isoprenoid artemisinin from FPP (Martin et al., 2003). Further analysis of these lines revealed the

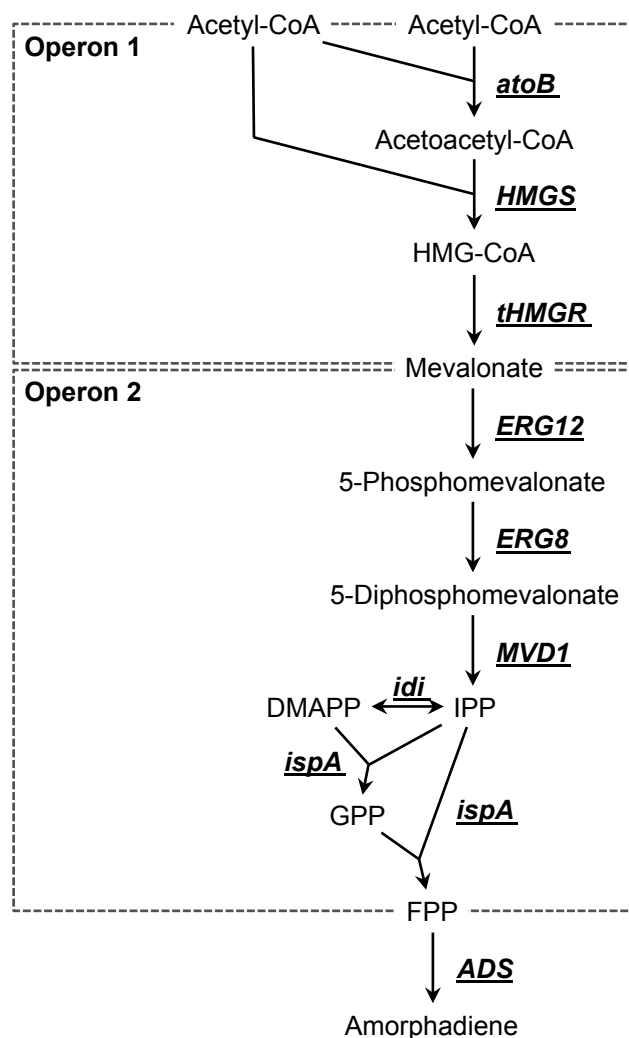


Figure 1.4. Mevalonate pathway genes expressed in *Escherichia coli* by Martin et al. (2003).

Genes (underlined) were from *S. cerevisiae* except where noted: ***ADS***, amorphaadiene synthase; ***atoB***, *E. coli* acetoacetyl-CoA thiolase; ***ERG12***, mevalonate kinase; ***ERG8***, 5-phosphomevalonate kinase; ***HMGS***, HMG-CoA synthase; ***idi***, *E. coli* isopentenyl diphosphate isomerase; ***ispA***, *E. coli* farnesyl diphosphate synthase; ***MVD1***, 5-diphosphomevalonate decarboxylase; ***tHMGR***, N-terminal truncated HMG-CoA reductase. Metabolites: **DMAPP**, dimethylallyl diphosphate; **FPP**, farnesyl diphosphate; **GPP**, geranyl diphosphate; **HMG-CoA**, 3-Hydroxy-3-methylglutaryl-CoA; **IPP**, isopentenyl diphosphate.

accumulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) (Pitera et al., 2007), which inhibited fatty acid synthesis (Martin et al., 2003; Kizer et al., 2008). Measuring amorphaadiene production by cells grown in various concentrations of mevalonate, along with the observation of HMG-CoA toxicity and knowledge of an abundant supply of acetyl-CoA in *E. coli*, enabled the discovery that HMG-CoA reductase (HMGR), which converts HMG-CoA into mevalonate, was significantly limiting flux into amorphaadiene (Pitera et al., 2007). Increasing enzyme production from operon one by optimisation of the intergenic regions (Pfleger et al., 2006), and increasing the number of copies of HMGR (Pitera et al., 2007), improved titres of amorphaadiene and recovered growth by limiting the accumulation of HMG-CoA.

Attaching pathway proteins to a synthetic scaffold made further improvements to mevalonate pathway flux. This was accomplished by adding protein-protein interaction domains into acetoacetyl-CoA thiolase (AtoB), HMG-CoA synthase and HMGR (Dueber et al., 2009). Highest titres (mevalonate production 77 times that achieved using the unscaffolded pathway) were achieved by assembling these proteins in a 1:2:2 ratio. The reasons for this ratio being more productive than a 1:1:1 ratio were not addressed by the authors but could be due to *E. coli* co-expressing a native AtoB or the AtoB possessing greater catalytic efficiency than HMG-CoA synthase and HMGR. This example demonstrates the improvements in product yield and decreases on the metabolic burden of enzyme synthesis that can be achieved by increasing the effective concentration of intermediates around the enzymes responsible for their turnover.

Later work used targeted proteomics to assess genes expressed at low levels in the transgenic *E. coli* (Redding-Johanson et al., 2011). This identified mevalonate kinase and phosphomevalonate kinase steps as exerting significant control over the engineered pathway. Promoter, codon and ribosome binding site optimisation by various means increased yields of

amorphadiene to 3.6 g l⁻¹, compared to 22.6 mg l⁻¹ in the initial strain (Redding-Johanson et al., 2011; Dahl et al., 2013; Nowroozi et al., 2014).

Similar work on amorphadiene biosynthesis has been performed in *S. cerevisiae* (Ro et al., 2006). In this system a limiting supply of acetyl-CoA to the mevalonate pathway was successfully alleviated by bypassing pyruvate dehydrogenase through the over-expression of acetaldehyde dehydrogenase and acetyl-CoA synthetase genes (Figure 1.5) (Shiba et al., 2007). Mevalonate and amorphadiene titres increased by 25% as a result.

Exploiting compartmentalisation

Subcellular compartmentation in plants complicates attempts at manipulating metabolite levels but also creates opportunities. As well as requiring that heterologous enzymes be targeted to the correct location of a specific biosynthetic pathway, the site of production can affect yields due to the availability of precursors, the pH of the compartment and the ability to bypass regulation of endogenous pathways located elsewhere. Subcellular targeting of engineered pathways may also prevent some of the pleiotropic effects of the accumulation of pathway intermediates (Vaughan et al., 2013). For instance, tobacco plants engineered to produce the mevalonate-derived artemisinin accumulated approximately eight times greater yields when amorphadiene synthase was targeted to the mitochondrion than when the gene was untargeted (Farhi et al., 2011a). In this and other examples (Farhi et al., 2011b), the success of the approach was thought to be due to higher availability of FPP in the mitochondrion than in the cytosol. A related approach involved transforming the tobacco plastidial genome with an entire heterologous mevalonate IPP biosynthetic pathway (Kumar et al., 2012). Not only did this increase carotenoid yields, but the largest observed isoprenoid yield increases were in squalene and the squalene-derived sterols, suggesting that excess prenyl diphosphates in the plastid were exported to the cytosol rather than being channelled into plastidial isoprenoids.

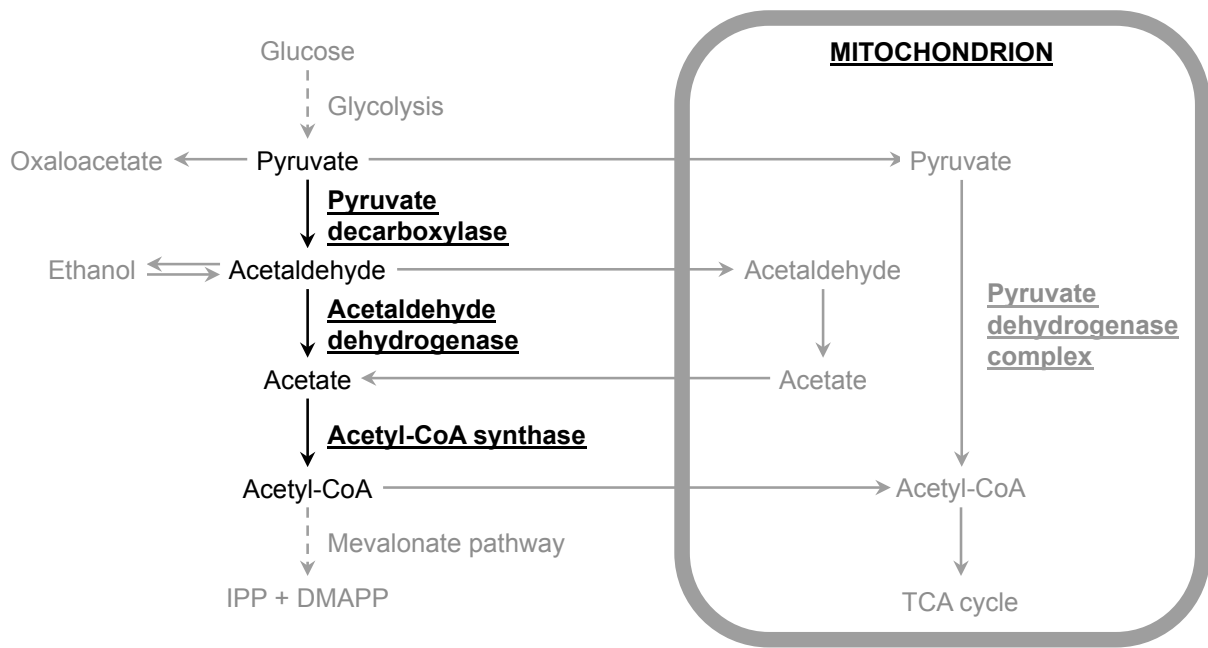


Figure 1.5. Schematic representation of the yeast pyruvate dehydrogenase bypass.

The pyruvate dehydrogenase bypass can convert pyruvate into acetyl-CoA in *S. cerevisiae* using pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthase (Pronk et al., 1996). Acetaldehyde dehydrogenase (*ALD6*) and acetyl-CoA synthase (*ACS1*) genes have been successfully over-expressed in *S. cerevisiae* to increase acetyl-CoA supply to the mevalonate pathway (Shiba et al., 2007).

1.2.5. Conclusions

Maximising secondary metabolite yields in plants is a complex process of identifying regulatory enzymes, adjusting their regulation through mutation or expression of heterologous genes, adjusting their production through manipulation of regulatory DNA sequences, considering where they should be targeted, and adjusting channelling through scaffolds. This requires targeted approaches where there exists an appropriate knowledge-base (such as in genomic information being available for FBA and knowledge of enzyme regulation) and trial and error (such as optimising expression parameters) where appropriate methods are available for the screening of many transgenic lines.

Work to date on metabolic engineering has highlighted the difficulty of predicting targets for increasing yields from a particular branch of metabolism. This is partly due to a lack of understanding about the control of metabolic flux in target pathways. It also highlights the problems that occur when depleting resources from essential competing pathways and from the build-up of toxic intermediates in unbalanced pathways, both of which limit growth and therefore absolute product yields. More recent work has shown the successes of optimising pathway flux to reduce the build-up of toxic intermediates. It has also shown that specific alterations to primary metabolism can increase yields of target secondary metabolites, particularly in bacteria, and that the success of some approaches is dependent on the prior implementation of other manipulations. Overall, the work indicates the current need for a more systematic approach to metabolic engineering in plants.

1.3. Sterol metabolism is an appropriate platform for investigating metabolic engineering in plants

Sterols are triterpenoid products of the mevalonate isoprenoid biosynthesis pathway. They are formed following reductive head-to-head condensation of two FPP molecules to form squalene, the first committed precursor for sterol biosynthesis (Figure 1.6). Sterols are products of membrane-localised enzymes (Hartmann and Benveniste, 1987). They are primarily localised in unmodified “free” form to the plasma membrane, where they function to regulate membrane fluidity and permeability (Schuler et al., 1991; Hartmann, 2004). The free 3 β -OH group faces the H₂O interface, while the side-chain extends into the hydrophobic core (Hartmann, 1998). Sterols are also precursors to the brassinosteroids (Fujioka and Sakurai, 1997) and are involved in the establishment of cell polarity through the targeting of auxin efflux proteins (Willemssen et al., 2003; Qian et al., 2013), the regulation of H⁺-ATPase activity (Grandmougin-Ferjani et al., 1997), cell expansion and division (He et al., 2003; Schrick et al., 2004), and are required for maintaining correct ploidy level during meiosis (De Storme et al., 2013).

Sterols are appropriate metabolites for the study of metabolic engineering in plants for several reasons. They are naturally synthesised in high quantities relative to many secondary metabolites, and therefore even relatively small increases in flux through sterol biosynthesis might have significant effects on the provision of substrate to competing reactions in either isoprenoid biosynthesis or further upstream in primary metabolism. As well as being endogenously synthesised in relatively large amounts, sterols are highly stable, as demonstrated by the increasing sterol-to-phospholipid ratio in senescing membranes (Duxbury et al., 1991). They can also be effectively made metabolically inactive by storage in

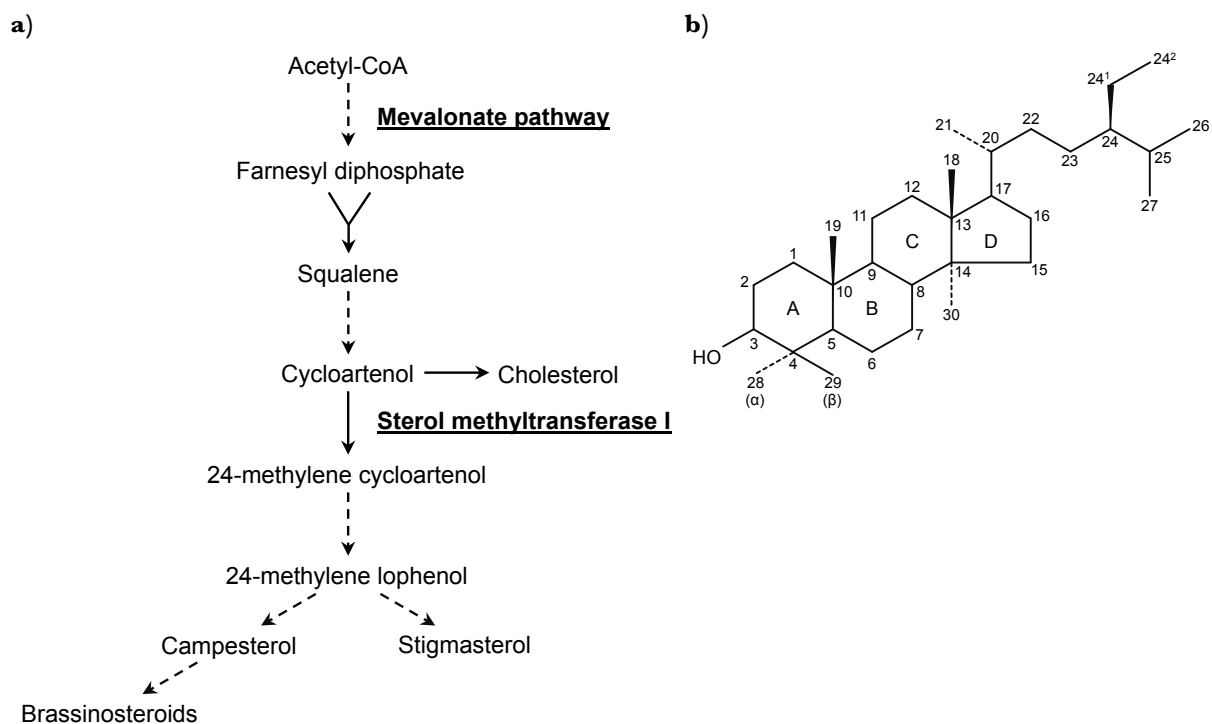


Figure 1.6. Sterol biosynthesis and structure.

a) Sterol biosynthesis from FPP. **b)** Sterol structure. Carbon numbering is according to International Union of Pure and Applied Chemistry and International Union of Biochemistry recommendations (IUPAC, 1989).

lipid vesicles following esterification or glycosylation of the 3 β -OH group (Gondet et al., 1994; Mongrand et al., 2010). These reactions occur with intermediate as well as end-of-pathway sterols (Hartmann, 2004), and thus allow concentration to be focused on the early steps of sterol biosynthesis.

Due to the intrinsic suitability of sterols for studying pathway genetic engineering, considerable research into the regulation of sterol metabolism has already been conducted in plants. This has led to the identification of HMGR (Chappell et al., 1995; Enfissi et al., 2005; Muñoz-Bertomeu et al., 2007) and sterol methyl transferase (Holmberg et al., 2002) as being enzymes exerting significant control over sterol formation.

1.4. Objectives and approach

The objective of this work was to increase sterol biosynthetic flux by manipulating the activity of enzymes within secondary metabolism (non-plastidial isoprenoid biosynthesis) and primary metabolism. It is believed that maximum yields of desirable secondary metabolites will only be achieved when the influence of the entire metabolic network on product yields is considered. Thus the goal was to demonstrate that specific enzymes in primary metabolism could exert significant control of flux into a specific group of secondary metabolites in plants, particularly when that branch of secondary metabolism has already been optimised.

Metabolic engineering of sterol biosynthesis was performed on tobacco (*Nicotiana tabacum*) Bright Yellow 2 cells. Attempts were made to increase the availability of acetyl-CoA to the mevalonate pathway by over-expressing a heterologous citrate synthase gene. The consequences of this on a range of parameters including the metabolome and the fluxome

were assessed to determine the success of the manipulation. Attempts were made to manipulate mevalonate pathway flux in tandem by manipulating HMGR activity by genetic and chemical means.

Chapter 2. Materials and methods

2.1. Chemicals

Lab consumables, unless otherwise stated, were purchased from Sigma-Aldrich Co. LLC (Poole, UK), Fisher Scientific UK Ltd (Loughborough, UK), VWR International Ltd (Lutterworth, UK) or Melford Laboratories Ltd (Ipswich, UK). H₂O was deionised using a MilliQ Water Ultra purification system (Merck Millipore, Darmstadt, Germany) and sterilised by autoclaving at 121°C for 20 min. The plant sterol mix was from Matreya, LLC (Pleasant Gap, Pennsylvania, USA).

2.2. Analytical equipment usage

2.2.1. Gas chromatography coupled to mass spectrometry

The gas chromatography coupled to mass spectrometry (GC-MS) system comprised an Agilent 7890A gas chromatography (GC) system with an Agilent VF5-ms column (30 m length, 0.25 mm inner diameter, 0.25 µm film) and an Agilent 7683 series injector, coupled to an Agilent 5975C quadrupole mass spectrometer (MS, Agilent Technologies UK Ltd, Wokingham, UK). The system was controlled using MSD ChemStation E.02.00.493 (Agilent Technologies UK Ltd.).

1 µl of sample was injected at 230°C and a 25:1 split ratio. GC parameters were varied depending on the derivatisation method used. The MS source and transfer line were maintained at 250°C, and the quadrupole was at 150°C. The MS was operated in scan mode

and monitored ions with mass-to-charge (m/z) ratios of 50-600 at 2.66 scans s^{-1} . MS tuning parameters were verified immediately prior to each run using perfluorotributylamine. GC-MS runs were interspersed with pyridine blanks to allow detection of carry-over between samples.

GC-MS data was baseline-corrected using MetAlign (Lommen, 2009) using the nominal mass mode with mass bin of 0.65. Retention scans 1-8,500 were corrected using a maximum amplitude of 10^9 , peak slope factor of $1.0 \times \text{noise}$, peak threshold factor of $2.0 \times \text{noise}$, and a peak threshold absolute value of 0. Average peak width at half height was set to five scans, and peak shapes were unaltered. Baseline-corrected data was analysed using MSD ChemStation E.02.00.493 and AMDIS 2.65 build 116.66 (National Institute of Standards and Technology, Gaithersburg, Maryland, USA).

Methoxyamine/trimethylsilyl derivatisation and analysis

Methanol extracts for metabolite analysis, as well as other extracts containing sterols or sugars, were derivatised with methoxyamine (MEOX) and trimethylsilyl (TMS) prior to GC-MS analysis (Lisec et al., 2006). Metabolite extracts were first dried using a speed-vac. Dried extracts were heated with 20 μl 20 mg ml^{-1} MEOX hydrochloride in pyridine for 2 h at 37°C with 900 rpm shaking. 40 μl *N*-methyltrimethylsilyltrifluoroacetamide (MSTFA; Regis Technologies, Inc., Morton Grove, Illinois, USA) was added and samples were heated at 37°C for a further 30 min. Derivatised samples were centrifuged at $16,000g$ for 10 min and then transferred to 200 μl glass GC-MS vials (Thermo Scientific Chromacol, Loughborough, UK) for analysis.

GC-MS parameters for separation of MEOX-TMS derivatives were based on those of Lytovchenko et al. (2009). A solvent delay of 10 min was used. Helium flow was $0.6 \text{ cm}^3 \text{ min}^{-1}$. After 5 min at 70°C the oven temperature was ramped to 350°C at 5°C min^{-1} . The

temperature was then dropped to 330°C for 5 min before being returned to 70°C at 120°C min⁻¹.

MEOX/TMS ethers were identified by comparison of mass spectra with a database of MEOX/TMS-derivatised plant metabolites (Kopka et al., 2005) using NIST MS Search 2.0c build 20041101 (National Institute of Standards and Technology) or by literature search.

***t*-butyldimethylsilyl derivatisation and analysis**

Derivatisation of amino acids and organic acids with *t*-butyldimethylsilyl (TBDMS) followed methods of Antoniewicz et al. (2007). Samples were dried using a speed-vac. Dried extracts were incubated at 37°C with 900 rpm shaking in 25 µl pyridine for 30 minutes after which 35 µl *N*-methyl-*N*-(*t*-butyldimethylsilyl) trifluoroacetamide containing 10 mg ml⁻¹ *t*-butyldimethylchlorosilane (Regis Technologies, Inc.) was added. Samples were then incubated for 30 minutes at 60°C with 900 rpm shaking. After derivatisation samples were centrifuged at 16,000*g* for 10 min. Supernatant was transferred to 200 µl glass vials for GC-MS analysis.

GC-MS parameters were similar to those used for analysis of MEOX/TMS derivatives with some exceptions. The oven temperature was initially set to 120°C for 5 min. This was then ramped to 270°C at 4°C min⁻¹. The temperature was held at 270°C for 3 min, ramped at 20°C min⁻¹ to 320°C and held for 1 min. Helium flow rate was 1.3 cm³ min⁻¹. Analytes were identified by comparison with a library of metabolite standard TBDMS ethers, created previously in our laboratory by Mr Pedro Bota.

2.2.2. ^1H nuclear magnetic resonance spectroscopy

^1H nuclear magnetic resonance spectroscopy (NMR) used a system comprising a Varian Unity Inova 600 NMR spectrometer (Varian, Inc.) with a 5 mm diameter $^1\text{H}\{^{13}\text{C}/^{15}\text{N}\}$ triple resonance probe (P6173_3res) and was operated using Solaris 2.6 (Varian, Inc.) and VNMRJ (Varian, Inc.) software. ^1H NMR spectra were recorded at 20 °C and 600 MHz. The acquisition used a 90° pulse (9 μs) and a 10 ppm spectral width. A pre-saturation delay of 2 s was used for water suppression, followed by a free induction decay (FID) of 4 s. Spectra were acquired for approximately 10 min. The FIDs were processed using NutsPro version 20070315 (Acorn NMR Inc., Livermore, California, USA). They were zero-filled, baseline-corrected and a line-broadening (exponential multiplication) of 1 Hz was applied. Automatic and manual phase correction was performed after Fourier transformation. This was followed by linear regression to flatten the spectral baseline.

2.2.3. Scintillation counting

Scintillation counting was performed using a Beckman LS 6500 multi-purpose scintillation counter (Beckman Coulter, Inc., High Wycombe, UK). Sample volumes were made up to 1 ml with H_2O and mixed with 4 ml of Optiphase HiSafe scintillation fluid (PerkinElmer Inc., Cambridge, UK). They were left overnight at room temperature to reduce the effect of chemiluminescence before disintegrations were measured over 5 min.

2.2.4. Oxygen electrode usage

Oxygen uptake assays were performed using a Hansatech Oxygraph oxygen electrode operated using Oxygraph V2.26 (Hansatech Instruments Ltd, King's Lynn, UK) at 25°C. Electrodes were calibrated at 100 kPa using H₂O. Approximately 200 mg Na₂S₂O₄ was added to remove O₂. Cell-free tobacco (*Nicotiana tabacum* L.) Bright Yellow 2 (BY-2) medium was used as a blank prior to assaying BY-2 oxygen uptake during exponential growth (typically four days after subculture). 0.5 ml culture samples were diluted 2x in cell-free medium and oxygen uptake was recorded over 2 min.

2.2.5. Epifluorescence microscopy

Epifluorescence microscopy was performed using a Leica MZ FLIII fluorescence stereomicroscope using green fluorescent protein (GFP) and green fluorescence filter sets for imaging GFP and mCherry, respectively. Images were captured with an Olympus DP71 camera, using Cell[^]A (Olympus Soft Imaging Solutions, Münster, Germany). Photographs were taken at 1 x magnification, with maximum aperture and an 8.6 s exposure.

2.3. Tobacco Bright Yellow 2 cell culture techniques

2.3.1. Bright Yellow 2 cell culture maintenance

BY-2 cell culture maintenance procedures were carried out in a category 2 laminar flow hood.

BY-2 culture medium contained 30 g l⁻¹ sucrose, 20 g l⁻¹ glucose or 30 g l⁻¹ glucose in addition to 4.3 g l⁻¹ Murashige and Skoog basal salt mixture and 200 µg l⁻¹ 2,4-dichlorophenoxyacetic acid. pH was adjusted to 5.2 with KOH prior to aliquoting and sealing with a double layer of aluminium foil. Medium was autoclaved in a Prestige Medical 2100/01 benchtop autoclave (Prestige Medical Ltd, Coventry, UK) with rapid heating and cooling to limit the Maillard reaction. When solid medium was required, 8 g l⁻¹ agar (Oxoid agar no. 3) was added before autoclaving. Antibiotics, when necessary, were added under sterile conditions after autoclaving the medium and allowing it to cool to approximately 45°C.

BY-2 cells were kindly provided by Professor Chris Hawes, Oxford Brookes University. Standard culture conditions were 50 ml suspension medium in 100 ml Erlenmeyer conical flasks on an orbital shaker at 130 rpm, 27°C in the dark. Approximately 500 mg fresh weight (FW) of cells, equating to 1.5 ml 20 g l⁻¹ glucose-grown wild-type cells, was transferred 7 days after subculture to fresh medium at room temperature using a cut-off 5 ml pipette tip.

BY-2 calli were maintained in the dark at room temperature on approximately 25 ml solid medium, with appropriate selective antibiotics, in 90 mm triple-vented petri dishes (Sterilin Ltd, Newport, UK) sealed with Parafilm. Approximately 4 mm² of callus was transplanted using a flame-sterilised wire loop to a new plate every two months, when the centre of the callus was greying but the periphery was still healthy.

2.3.2. Growth analysis

1.5 ml culture suspension was aliquoted into pre-weighed 1.5 ml microfuge tubes using a cut-off 5 ml pipette tip at 24 h intervals. Cells settled by centrifugation at 2,300g for 3 min. The

majority of the culture medium was removed by pipetting. Centrifugation was repeated followed by removal of the remainder of the culture medium. Care was taken not to remove settled cells. Microfuge tubes were re-weighed on a fine balance (accurate to 0.1 mg) allowing calculation of FW. For measurements of dry weight (DW), samples were snap-frozen on liquid nitrogen, lyophilised and re-weighed.

2.3.3. Harvesting of plant material

BY-2 cell cultures were harvested by vacuum filtration through Whatman No. 4 filter paper (Whatman Ltd, Maidstone, UK) in a Büchner funnel until the compacted cells had formed a dry cake. Harvested cells, tobacco leaves and culture filtrate (CCF) were frozen on liquid nitrogen. Cells and leaves were ground on liquid nitrogen in a pestle and mortar. When dry material was required, samples were dried by lyophilisation.

2.3.4. Cell viability

Cell viability was determined using Evans blue stain, which is excluded from intact cells but accumulates in the protoplasm of dead cells (Turner and Novacky, 1974). 2 µl of 200 mg ml⁻¹ Evans blue stain was added to 1 ml cell suspension. Cells were allowed to stain for 3 min at room temperature before mounting 20 µl onto microscopy slides. Photographs were taken of slides from different cultures. Cell viability was a measure of the number of nuclei from unstained cells divided by the sum of the number of nuclei from unstained cells and the number of stained cells.

2.4. Metabolite extraction and analysis

2.4.1. Sterol extraction and analysis

Hexane extraction of total sterols from BY-2 cells and tobacco leaves was based on Fraser et al. (2007). This used KOH to hydrolyse sterol esters, which, unlike acid hydrolysis, retains the integrity of Δ^5 - and Δ^7 -avenasterol (Kesselmeier et al., 1985; Kamal-Eldin et al., 1998). 5 ml of 5 mg ml⁻¹ KOH in 80% (v/v) ethanol was added to 50 mg ground, dried plant material. Samples were mixed on a vortex and heated for 1 h at 80°C. They were then transferred to ice and pH was adjusted to 6-8 using concentrated H₂SO₄ and KOH. Sample volumes were adjusted to 7 ml with H₂O. Sterols were extracted three times by adding 3 ml hexane, mixing by vortex, and centrifuging at 1,000g for 1 min. The upper hexane fractions were stored for up to two weeks at -20°C. For extraction of free sterols, KOH was omitted from the initial extraction step.

For analysis by GC-MS, 6 µg nonadecanoic acid was added to volumes of hexane extract equating to 3.75 mg DW BY-2 cells. These extracts were then derivatised using MEOX and TMS. Sterols were quantified relative to nonadecanoic acid. GC peaks were integrated using the default settings in MSD ChemStation (initial area reject = 0; initial peak width = 0.040; shoulder detection = off; initial threshold = 13.0).

¹H-NMR was performed on dried plant sterol mix or BY-2 total sterol extract resuspended in 800 µl CDCl₃. Chemical shifts were measured relative to the CHCl₃ signal at 7.26 ppm. Peak abundances were obtained by manual integration. Chemical shift values were searched

against published chemical shifts of sterols in CDCl_3 measured with respect to the CHCl_3 signal at 7.26 ppm.

2.4.2. Acetyl coenzyme A extraction and analysis

Acetyl coenzyme A (acetyl-CoA) extraction was based on a method developed by Sweetlove et al. (1996). 0.2 g frozen tissue was ground in 1 ml ice-cold 16% (w/v) trichloroacetic acid until a homogenate was formed. This was incubated on ice for 2 h to precipitate proteins. Samples were then centrifuged at 16,000g for 5 min at 4°C. The supernatant was transferred to a new microfuge tube, and the trichloroacetic acid was removed three times using an equal volume of H_2O -saturated diethyl ether. This was done by vortexing for 10 s, centrifuging at 16,000g, 4 °C, for 10 s, and discarding the upper diethyl ether phase. 100 μl of 1 M sodium acetate (pH 5.5) was added to the aqueous phase. Microfuge tubes were left uncapped, on ice, in a fumehood to allow any remaining ether to evaporate.

An expected low *in vivo* concentration precluded analysis of acetyl-CoA by GC-MS. Instead it was determined by the rate of increase in A_{340} as NAD^+ is reduced in an enzymatic cycling assay (Gibon et al., 2002; Figure 2.1). 20 μl volumes of acetyl-CoA extract were analysed in 96 well microtitre plates. CoA-SH was removed by incubation with 20 μl of 50 mM *N*-ethylmaleimide at room temperature for 10 min. 100 μl of 200 mM tris(hydroxymethyl)aminomethane (Tris) HCl (pH 7.4) containing 1 μmol dithiothreitol (DTT) and 20 μmol malate was then added and samples were incubated for a further 15 min to thiolate the *N*-ethylmaleimide. 50 μl of a solution containing 0.5 μmol DTT, 0.92 μmol acetyl phosphate, 0.28 μmol β -nicotinamide adenine dinucleotide hydrate, 2.8 U

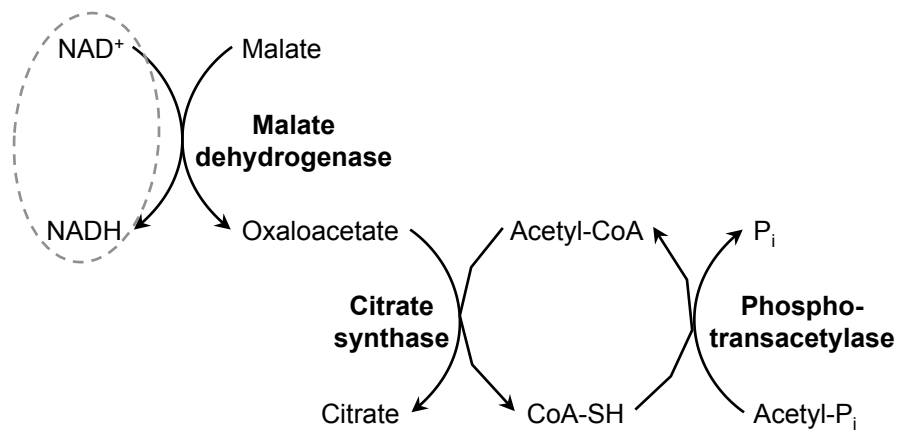


Figure 2.1. Quantification of acetyl-CoA using an enzyme cycling assay.

Acetyl-CoA was quantified by measuring the rate of increase in A_{340} as β -nicotinamide adenine dinucleotide (NAD^+) is reduced. Acetyl-CoA is limiting when excess β -nicotinamide adenine dinucleotide hydrate, malate (L-malic acid) and acetyl- P_i (acetyl phosphate) are supplied.

phosphotransacetylase, 1 U citrate synthase (CS) and 2 units mitochondrial L-malate dehydrogenase from pig heart (Roche Diagnostics Ltd, Burgess Hill, UK) in Tris-HCl 100 mM was then added, and absorbance at 340 nm was measured at 20 s intervals for 10 min with a Beckman Coulter DTX880 Multimode Detector (microplate reader) operated using Multimode Analysis Software 3.3.0.9 (Beckman Coulter, Inc.).

2.4.3. Capsidiol extraction and analysis

Capsidiol was extracted from harvested cells and CCF using methodology based on that of Wibberley et al. (1994). Harvested cells were homogenised with ice-cold 2.5 ml (g FW)⁻¹ of 2% (v/v) NH₄OH. Capsidiol was extracted twice from homogenised cells or CCF using ice-cold CHCl₃ in a 1:1 ratio. Samples were mixed by vigorously shaking, centrifuged at 3,500g for 10 min at 4°C, and the lower CHCl₃ was transferred to a fresh tube. CHCl₃ extracts were dried using a speed-vac. Dried samples were resuspended in 20 µl methanol and centrifuged at 16,000g for 10 min. The supernatant was transferred to GC-MS vials for analysis. 10% (v/v) 0.5 mg ml⁻¹ pentadecane in pyridine was added as an internal standard. GC-MS parameters were as for MEOX/TMS-derivatised samples with the exception that the solvent delay was extended to 15 min to avoid saturation of the detector by methanol. An authentic capsidiol standard (Cfm Oskar Tropitzsch, Marktredwitz, Germany) was dissolved in methanol to a concentration of 0.1 mg ml⁻¹ and analysed similarly.

2.4.4. Methanol extraction and analysis of organic acids, amino acids and sugars

Methanol-soluble metabolites were extracted from 100 mg lyophilised cells (Masakapalli et al., 2010). This was done by first adding 60 μl of 0.2 mg ml^{-1} ribitol as an internal standard to each sample and then thawing on ice for 2 h with 5 ml ice-cold methanol. Samples were then vortexed vigorously and centrifuged at 2,050g for 30 min at 4 °C. Supernatant was removed and stored on ice. The pellet was resuspended in 5 ml ice-cold methanol, vortexed, and incubated on ice for 5 min before centrifugation as described and combination of the supernatants. The pellet was again suspended in 5 ml ice-cold methanol. This time it was incubated at 70 °C for 5 min. The suspension was cooled on ice before centrifugation as before and combining the supernatant with those of the previous steps. When desirable, the pellet from this step was stored at -20 °C for starch and protein extraction.

Fatty acids were extracted from methanol extracts by adding CHCl_3 and H_2O to the methanol extracts to generate a $\text{CHCl}_3\text{:CH}_3\text{OH:H}_2\text{O}$ mixture of 1:2:2 (v/v/v). This was vortexed and centrifuged at 3,500g for 10 min. The aqueous methanol phase was partially dried by rotary evaporation at 37.5 °C and resuspended to a total volume of 3 ml in H_2O .

Comparative analyses of methanol-soluble metabolites

Appropriate volumes of methanol extracts were dried using a speed-vac for derivatisation using MEOX/TMS and GC-MS analysis. 10 μl of an alkane mix containing linear alkanes of 10, 12, 15, 18, 19, 22, 28, 32 and 36 C, each at 22 $\mu\text{g ml}^{-1}$, was added with MSTFA during

the derivatisation process as retention time standards to allow identification of analytes in different samples simultaneously using TagFinder (Luedemann et al., 2008).

Statistical analysis of metabolomic data involved analysis of variance using StatPlus:mac 5.8.2.0 (AnalystSoft Inc.) and multivariate analysis of variance using MATLAB 7.11.0.584 (R2010b, The Mathworks Inc.). This returned p-values based on Wilks' λ . Principal component analysis and partial least squares projection to latent structures were also performed using SIMCA 13.0.2 (Umetrics Ltd). Values of dependent variables were mean-centred and scaled to unit variance to emphasise variables equally regardless of their absolute values or variance.

Assaying citrate and isocitrate

30 μ l resuspended methanol extract was used for citrate and isocitrate quantification. 1 μ g norvaline (1 mg ml⁻¹ in 0.1 M HCl) was added as an internal standard prior to drying the extract by speed-vac, derivatising with MEOX and TMS, and GC-MS analysis.

2.5. Enzyme assays

2.5.1. Extraction of total BY-2 protein

BY-2 proteins were extracted at 4°C using a buffer containing 50 mM HEPES-KOH (pH 7.3), 5 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 2 mM benzamidine hydrochloride hydrate and 2 mM ϵ -aminocaproic acid. 5 mM DTT was added immediately prior to extraction. Five 5 ml volumes of this buffer were used to equilibrate PD-10 desalting

columns (GE Healthcare, Little Chalfont, UK), before 0.5 mM phenylmethylsulphonyl fluoride was added. Ground BY-2 cells were suspended in 10 μ l (mg FW)⁻¹ of this extraction buffer. Suspended cells were centrifuged at 18,800g for 10 min at 4°C. 2.5 ml supernatant was then applied to the equilibrated column. After absorption into the packed bed, proteins were eluted using 3.5 ml extraction buffer and snap-frozen on liquid nitrogen. Columns were rinsed for re-use using H₂O.

2.5.2. Extraction of BY-2 microsomal protein

Microsomal protein was extracted from ground cells at 4°C using a method adapted from Hemmerlin and Bach (2000). All equipment used during microsomal extraction was free of detergent. Cells were cultured in detergent-free medium and weighed after harvesting, immediately prior to grinding on liquid nitrogen. Ground cells were homogenised by shaking vigorously in 12.5 ml (g FW)⁻¹ extraction buffer containing 0.2 M KH₂PO₄ (pH 7.5), 0.35 M sorbitol, 10 mM Na₂EDTA, 5 mM MgCl₂ x 6H₂O, 20 mM dithioerythritol and 0.04 g ml⁻¹ polyvinylpyrrolidone (average M_r = 40,000). Cell debris was filtered through 50 μ m nylon gauze and then centrifuged in approximately 50 ml volumes for 5 min at 1,500g. The supernatant was then centrifuged for 40 min at 16,000g. Approximately 20 ml supernatant from this step was centrifuged for 1 h at 105,000g. Pellets were resuspended using a paintbrush in approximately 20 ml resuspension buffer containing 0.2 M Tris-HCl (pH 7.5) and 20 mM DTT. Samples were centrifuged again for 1 h at 105,000g. Pellets were resuspended in approximately 20-50 μ l resuspension buffer using a cut-off 200 μ l pipette tip and were snap-frozen on liquid nitrogen.

2.5.3. Quantification of protein extracts

Protein extracts were analysed in triplicate in 1, 2, 5 or 10 µl volumes using a Bio-Rad protein assay (Bio-Rad Laboratories Ltd., Hemel Hemstead, UK) according to instructions. Absorbance at 595 nm was measured in 200 µl volumes in 96 well microtitre plates at room temperature using a microplate reader. Protein was quantified by comparison with A₅₉₅ values from a dilution series of bovine serum albumin (BSA).

2.5.4. Citrate synthase assay

CS was assayed at room temperature by means of a coupled assay (Stitt et al., 1989, Figure 2.2). Approximately 100 µg protein extract was analysed in 1 ml cuvettes (1 cm light-path) with 100 mM triethanolamine, 0.5% (v/v) Triton X-100, 10 mM L-malic acid disodium salt, 0.2 mM acetylpyridine adenine dinucleotide (APAD), 12 U L-malate dehydrogenase and 0.2 mM acetyl-CoA. Reduction of APAD was followed by measurement of A₃₆₅ at 1 min intervals using a Unicam UV4 UV/Vis spectrometer operated using Vision 3.40 (Thermo Spectronic, Cambridge, UK). CS content was calculated by comparing the rate of A₃₆₅ increase after addition of acetyl-CoA with the rate before. Acetyl-CoA consumption (mM min^{-1}) = $\Delta A_{365} \text{ min}^{-1} / 9.1$.

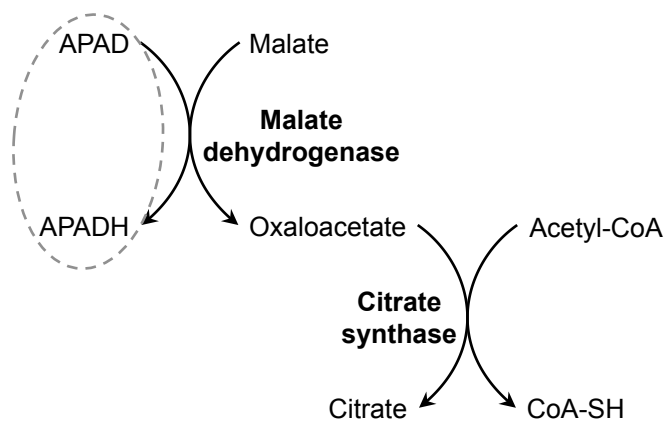


Figure 2.2. Quantification of citrate synthase activity using a coupled assay.

Synthesis of citrate from oxaloacetate and acetyl-CoA was coupled to synthesis of oxaloacetate from malate using APAD as a cofactor (Stitt et al., 1989). CS activity is proportional to the rate of increase in A_{365} as APAD is reduced.

2.5.5. Spectrophotometric 3-hydroxy-3-methylglutaryl coenzyme A reductase assay

3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyses the irreversible reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate using NADPH as a cofactor (Brooker and Russell, 1975; Bach and Lichtenthaler, 1984; Omkumar and Rodwell, 1994). The oxidation of NADPH allows HMGR activity to be assayed by the rate of the decrease in A_{340} (Asadollahi et al., 2010). Reactions were either performed in 1 ml volumes in 1 ml cuvettes (1 cm light-path) or in 200 μ l volumes in 200 μ l 96 well microtitre plates. The reaction mixture comprised 100 mM KPO_4 , pH 7.0, 0.5% (v/v) Nonidet P-40, 5 mM DTT, 150 μ M NADPH and a variable quantity of BY-2 total protein or microsomal protein extract. 1 ml samples were analysed using a Unicam UV4 UV/Vis spectrometer with A_{340} measured at 1 min intervals. A_{340} of 200 μ l samples was measured at 30 s intervals using a microtitre plate reader. After 3 min HMG-CoA was added to a total concentration of 150 μ M. The rate of A_{340} increase was subtracted from the rate prior to addition of HMG-CoA. HMG-CoA reduction ($mM\ min^{-1}$) was calculated as $(\Delta A_{340}\ min^{-1}) / 6.22$.

2.5.6. ^{14}C 3-hydroxy-3-methylglutaryl coenzyme A reductase assay

The ^{14}C HMGR assay is based on that of Enfissi et al. (2005). Approximately 30 μ g microsomal protein was used. Assays were performed in 30 μ l volumes containing 0.1 M Tris-HCl (pH 7.5), 10 mM DTT, 4.3 mM NADPH and 67 μ M, 1.94 GBq.mmol $^{-1}$ DL-3-[glutaryl- 3- ^{14}C]-hydroxy-3-methylglutaryl coenzyme A (PerkinElmer Inc.). Assay mixtures were incubated for 30 min at 27°C. 10 μ l 6 M HCl was added to stop the reaction.

After 15 min at room temperature samples were centrifuged at 10,000*g* for 1 min. 15 µl of each sample was applied to a 20 cm x 20 cm aluminium-backed 250 µm silica G thin layer chromatography (TLC) plate (Whatman Ltd, Maidstone, UK) and developed with 3:1 (v/v) diethyl ether/acetone.

TLC plates were used to expose phosphorimaging screens for 24 h. Phosphorimaging screens were scanned using a Bio-Rad Molecular Imager FX operated using Quantity One 4.4.1 build 067 (Bio-Rad Laboratories, Inc.). Phosphorimaging results were used to locate radioactivity on the TLC plates for purposes of scintillation counting. TLC plates were cut up and silica gel was scraped into 5 ml scintillation vials for this purpose.

2.6. Creation of transgene constructs and transformation of BY-2 cells

2.6.1. *Escherichia coli* culture

E. coli DH5α and DB3.1 were cultured either in suspension at 37°C with 200 rpm shaking in sterile lysogeny broth (LB) (Bertani, 1951) or at 37°C on sterile LB agar in 90 mm triple-vented petri dishes sealed with Parafilm. LB and LB agar were prepared by Mr Pedro Bota and Ms Rita Ross. Culture stocks, made by adding 800 µl culture to 200 µl glycerol and snap-freezing on liquid nitrogen, were stored at -80°C.

2.6.2. Preparation of chemically competent *Escherichia coli* DH5 α

E. coli DH5 α were made competent following a method based on Sambrook et al. (1989) and Tang et al. (1994). 4 ml sterile LB was inoculated with 50 μ l chemically competent *E. coli* DH5 α and grown overnight. 100 ml LB was then inoculated with 1 ml of the overnight culture and grown to an OD₆₀₀ of 0.4. The culture was split between two 50 ml conical centrifuge tube and incubated on ice for 10 min. The culture was then centrifuged at 1,000g and 4°C for 10 min. The supernatant was discarded and pellets were resuspended in 5 ml ice-cold filter-sterilised transformation buffer containing 60 mM CaCl₂, 10 mM PIPES-HCl, pH 7.0 and 15% (v/v) glycerol by gentle pipetting. Resuspended pellets were centrifuged as previously and supernatant was discarded. Pellets were each resuspended in 5 ml ice-cold transformation buffer, incubated on ice for 1 h and centrifuged as previously. Pellets were resuspended in 1 ml ice-cold transformation buffer, aliquoted in 50 μ l volumes, snap-frozen on liquid nitrogen and stored at -80°C.

2.6.3. Purification of plasmid vectors for tobacco BY-2 transformation

pH7WG2D and pK7WG2 plasmids (Karimi et al. (2002), acquired from VIB Department of Plant Systems Biology, University of Gent), and pDONR201 (Invitrogen, Life Technologies Corporation, Paisley, UK) were purified from *E. coli* DB3.1 grown in 5 ml LB containing either 100 μ g ml⁻¹ spectinomycin (pH7WG2D and pK7WG2) or 50 μ g ml⁻¹ kanamycin (pDONR201) using a QIAprep Spin Miniprep kit (QIAGEN GmbH). 4 ml LB culture was centrifuged at 10,000g for 3 min. The pellet was then processed according to the protocol “Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge” in

the QIAprep Miniprep Handbook. Purified plasmids were resuspended in 50 µl H₂O and stored at -20°C. 1 µl was verified by agarose gel electrophoresis (AGE) and the DNA concentration was determined using a NanoDrop 1000 (Thermo Fisher Scientific Inc., Loughborough, UK). A 1:100 dilution was used as a polymerase chain reaction (PCR) template.

2.6.4. Transformation of *Escherichia coli*

Plasmid DNA was added to competent *E. coli* and incubated on ice for 30 min. Cells were heat-shocked at 42°C for 30 s and 250 µl super optimal broth with catabolite repression (prepared by Mr Pedro Bota and Ms Rita Ross) was added. Cells were then incubated at 37°C for 1 h with 200 rpm shaking. 10-200 µl was spread onto LB agar with appropriate antibiotics using a flame-sterilised glass spreader. Plates were sealed with Parafilm and incubated at 37°C overnight. Resultant colonies were screened by polymerase chain reaction (PCR) of 1 µl of a 30 µl dilution in H₂O. The remaining colony dilutions were grown overnight in 5 ml LB containing appropriate antibiotics.

2.6.5. Polymerase chain reaction

Dried salt-free oligonucleotide primers (Eurofins MWG Operon, London, UK), designed using FinchTV 1.4.0 (Geospiza Inc., Seattle, Washington, USA), were resuspended to 100 µM in H₂O. These stocks and 10 µM working stocks were stored at -20°C. A Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany) was used for PCR.

Analytical PCRs used 1 µl template, 0.5 µl (5 pmol) each primer and 15 µl MegaMix-Blue (Microzone Ltd, Haywards Heath, UK). Initial denaturing was performed at 3 min at 94°C followed by 35 cycles of 94°C for 20 s, annealing at approximately 5°C below the melting temperature listed in Table 2.1 for 30 s, and extension at 72°C for 1 min kb⁻¹. An additional 5 min elongation at 72°C was added to the final cycle. 10 to 15 µl was analysed by AGE.

PCR for transferring DNA into plasmids was performed in 50 µl volumes containing 1 x Phusion high-fidelity reaction buffer with 1-3 µl of template, 0.1 nM each primer, 0.2 µM dNTPs and 1 U Phusion high-fidelity DNA polymerase. Each reaction had an initial denaturation step at 98°C for 30 s. Other steps varied between templates.

2.6.6. Restriction digestion

Restriction digests were performed using FastDigest enzymes (Thermo Fisher Scientific Inc.) according to the supplied protocols.

2.6.7. Agarose gel electrophoresis

PCR products, restriction digest products and purified plasmids were run on 1% (w/v) agarose gels containing 40 mM Tris-acetate, 1 mM EDTA and 1 µM ethidium bromide. Samples were added to 2 µl loading buffer except for when a loading dye was already present in the reaction buffer. 2-3 µl HyperLadder 1 (Bioline Reagents Ltd) was used as a ladder.

Table 2.1. PCR primer sequences.

Name	Sequence (5'→3')	Bases	GC content (%)	Melting temperature (°C)
At-Hmg1-F	CATTCCTCCAATGGATCTC	20	50	59.3
At-Hmg1-R	TGATTCAGATTCAGATCATGTT	23	34.8	61.5
ATG_At-tHmg1-F	G ATGGTTACCGAATCGCTTCCTG	22 (19)	50 (52.7) ¹	62.2 (64.5)
CS-OX-F	TGAAGCTCTCGAATCTCTGG	20	50	58.3
CS-OX-R	TGGTGACTTAAGCAGATGAAGC	22	45	59.5
GW1	GTTGCAACAAATTGATGAGCAA	25	40	59.7
	TGC			
GW2	GTTGCAACAAATTGATGAGCAA	25	32	56.4
	TTA			
mCherry-F	ATGGTGAGCAAGGGCGAG	18	61.1	58.2
mCherry-R	TTACTTGACAGCTCGTCCATG	23	47.8	60.3
	C			
p7WG2-ins-F	GTTCAATTCATTTGGAGAGGAC	22	40.9	57.6
p7WG2-ins-R	GCGGGATATCACCCTTTG	19	52.6	58.9
pBI121-ins-F	CACAATCCCCTATCCTTCG	20	50	57.3
pBI121-ins-R	ACGATCGGGGAAATTCGAG	19	53	56.7
pBI121-ins-R2	AGGCGATTAAGTTGGGTAACG	21	48	57.9
pBI121-T-DNA-F	GCACATACAAATGGACGAACG	21	48	57.9
pBI121-T-DNA-R	AAAGTCTGCCGCCCTTACAAC	20	50	57.3
pUB-ins-F	CTGGTGTTAGTTTCTAGTTTGT	24	41.7	57.3
	GC			
pUB-ins-R	TTGCGGACTCTAGCATGG	18	55.6	58.5
T35S-R	TCCCTTATCGGGAACTACTCA	22	45	58.4
Truncated_At-Hmg1-F	ATGGTTACCGAATCGCTTCCT	21	47.6	62

¹ATG_At-tHMG1-F was used to amplify the truncated *Hmg1* coding sequence from the pBI121-*Hmg1* vector. The 5' ATG bases were introduced by the primer, and did not anneal to the template. The base number, GC content and T_m in brackets applies to the section of primer that anneals to the pBI121-*Hmg1* template.

Agarose gels were run at 7 V cm⁻¹ for approximately 20 min before being photographed under UV light.

2.6.8. DNA sequencing

Plasmids were Sanger sequenced by Source Bioscience UK Ltd. 5 µl 100 ng µl⁻¹ purified plasmid DNA was submitted for each reaction along with 5 µl 3.2 pmol µl⁻¹ primer.

2.6.9. Creation of BY-2 expression vectors

Sequences of all primers referenced in this section are provided in Table 2.1.

Amplification of *mCherry*

mCherry (Shaner et al., 2004) was amplified from 1 µl of a 1:100 dilution of purified pRSET-B (kindly provided by Dr Monika Kalde) using mCherry-F and mCherry-R primers. Thermocycler settings were 35 cycles at 98°C for 10 s, primer annealing at 52°C for 30 s, and 72°C extension for 45 s. The final cycle had an extra 15 min elongation. The thermocycler lid was maintained at 104°C. A reaction product of approximately 0.7 kb was confirmed by AGE of 10 µl.

Amplification of *Arabidopsis thaliana* CITRATE SYNTHASE 4

The open reading frame of *Arabidopsis thaliana* CITRATE SYNTHASE 4 (At2g44350; *ATCS*) nuclear gene encoding a mitochondrion-localised CS was amplified from 3 µl *A. thaliana*

complementary DNA (cDNA) from a cDNA library using primers CS-OX-F and CS-OX-R. 35 cycles of PCR were used with denaturation at 98°C for 10 s, primer annealing at 55°C for 30 s, and 72°C extension for 45 s. The final cycle had an extra 10 min elongation. The thermocycler lid was maintained at 108°C. A reaction product approximating 1.5 kb was confirmed by AGE of 5 µl product.

Verification and amplification of *Arabidopsis thaliana* 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE 1

The *A. thaliana* 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE 1 (At1g76490; *HMG1*) cDNA was kindly provided in the pBI121 plant expression vector by Dr Eugenia Enfissi (Royal Holloway University of London). *E. coli* were transformed with this construct and grown on 50 µg ml⁻¹ kanamycin LB agar. Purified plasmids were initially verified by EcoRI digest (giving bands of 2.3 kb and 13 kb on agarose gel) and by analytical PCR using the primers pBI121-ins-F and pBI121-ins-R (giving a 2.5 kb product). PCR parameters were 3 min at 94°C followed by 35 cycles of 94°C for 20 s, 50°C for 30 s, and 72°C for 2 min 40 s. An additional 5 min elongation at 72°C was added to the final cycle. Sequencing with primers pBI121-ins-F, pBI121-ins-R and pBI121-ins-R2 provided final confirmation.

PCR amplification of *HMG1* and truncated *HMG1* (*tHMG1*) for transfer to pUB-DEST (Grefen et al., 2010) used primers At-HMG1-F and At-HMG1-R, and ATG_At-HMG1-F and At-HMG1-R, respectively. Each used 1 µl of a 1:100 dilution of purified pBI121-*HMG1* as template. 28 thermocycles were used with 98°C denaturation for 10 s, 54°C annealing for 30 s and extension at 72°C for 30 s. The final extension step was extended by 5 min.

T/A cloning of PCR products into pCR8/GW/TOPO

mCherry, *ATCS*, *HMG1* and *tHMG1* PCR products were separately ligated into the pCR8/GW/TOPO Gateway entry vector (Invitrogen). First, adenosine overhangs were added to the 3' ends by incubating 0.4 μ l (2 U) Taq DNA polymerase from *Thermus aquaticus* (Sigma-Aldrich Co. LLC Poole, UK) with the remaining PCR reaction volume for 10 min at 72°C. 2 μ l of this reaction mixture was added to 0.5 μ l pCR8/GW/TOPO and 0.5 μ l TOPO cloning salt solution (Invitrogen, Life Technologies Corporation) and incubated at room temperature for 8 min, according to the pCR8/GW/TOPO TA Cloning Kit instructions. Competent *E. coli* DH5 α were transformed with the reaction product and spread on 100 μ g ml⁻¹ spectinomycin LB agar.

Analytical PCR followed by AGE was performed to confirm presence and orientation of the insert by using combinations of the primers used to initially amplify the genes and primers GW1/GW2. Suspension cultures were grown with cells containing the plasmids in the correct orientation and plasmids were purified. Sequencing with GW1 and GW2 primers provided verification of insert orientation.

Transfer of transgenes to Gateway destination vectors

Due to pCR8/GW/TOPO and the desired destination vectors each conferring spectinomycin resistance to *E. coli*, transgenes were transferred to the intermediary vector pET-DEST42 followed by pDONR201. These confer resistance to 100 μ g ml⁻¹ ampicillin and 50 μ g ml⁻¹ kanamycin, respectively. Transgene inserts was transferred from pCR8/GW/TOPO using LR clonase II and BP clonase II, respectively, according to the Gateway Technology manual (Invitrogen, Life Technologies Corporation), but with reaction volumes reduced to 5 μ l. BglIII was used to linearise pET-EXPR42-*mCherry* and pET-EXPR42-*ATCS* constructs (resulting from transgene transfer to pET-DEST42), and AccI was used to linearise

pET-EXPR42-*HMG1* and pET-EXPR42-*tHMG1* prior to the BP clonase II reaction. At each stage, the presence of the insert and identity of the vector were confirmed by colony PCR and analytical digestion.

An LR clonase II reaction was used to transfer transgenes from pENTR201 (arising from transgene insertion into pDONR201) to destination vectors. *E. coli* DH5 α colonies arising from transformation with the reaction product were screened for the insert by colony PCR with primers amplifying the insert coding region. Different primer sets were used to confirm the presence of the insert in the destination vector after purifying plasmids. These primer sets comprised a) forward and reverse primers annealing to the insert only, b) forward primer annealing to the insert and p7WG2-ins-R, which anneals immediately downstream of the 3' Gateway recombination site, and c) p7WG2-ins-F, which anneals immediately upstream of the 5' Gateway recombination site, and the insert reverse primer. Products were expected from each reaction. pH7WG2D-*mCherry*, pK7WG2-*mCherry* and pH7WG2D-*ATCS* expression vector inserts were confirmed by sequencing using primers p7WG2-ins-F and p7WG2-ins-R. pUB-EXPR-*mCherry* sequencing used primer pUB-ins-F. pUB-EXPR-*HMG1* was sequenced with pUB-ins-F and pUB-ins-R. pUB-EXPR-*tHMG1* was sequenced with pUB-ins-F and Truncated_At-HMG1-F.

2.6.10. Chemically competent *Agrobacterium tumefaciens* GV3101 (pMP90)

preparation

Agrobacterium tumefaciens GV3101 (pMP90) was spread on LB agar containing 25 $\mu\text{g ml}^{-1}$ gentamycin and grown overnight at 28°C. A single colony was picked and grown overnight in 5 ml LB containing 25 $\mu\text{g ml}^{-1}$ gentamycin at 28°C with 170 rpm shaking. This culture was

used to inoculate 100 ml LB containing 25 $\mu\text{g ml}^{-1}$ gentamycin and was grown at 28°C with 170 rpm shaking until an OD₆₈₀ of 0.5-1.0 was achieved. The culture was chilled on ice and then centrifuged briefly at 440g to remove unwanted clumps of cells. The cell suspension was centrifuged at 1,750g for 6 min at 4 °C. The supernatant was discarded and the pellet was gently resuspended by pipetting in 1.4 ml of ice-cold filter-sterilised 20 mM CaCl₂. 200 μl aliquots were frozen on liquid nitrogen and stored at -80°C.

2.6.11. Transformation of agrobacteria

Competent agrobacteria were thawed with 10 μl (0.5-2 μg) purified plasmid at 37°C for 5 min. 1 ml LB was added and a suspension made by gentle pipetting. Incubation at 28°C with 150 rpm shaking for 4 h followed. Cells were then centrifuged at 16,000g for 30 s. Pellets were resuspended in 100 μl LB and spread onto LB agar containing 25 $\mu\text{g ml}^{-1}$ gentamycin and the appropriate antibiotic to select against untransformed agrobacteria. Plates were sealed with Parafilm and incubated for two to three days at 28°C.

2.6.12. *Agrobacterium*-mediated transformation of BY-2 cells

All steps of the transformation procedure were carried out in a category two laminar flow hood. Transformation was performed as described by Brandizzi et al. (2003). Agrobacteria were washed to remove antibiotics by centrifuging 1 ml overnight suspension culture at 6,200g for 3 min at room temperature, discarding the supernatant, and resuspending the pellet in 1 ml BY-2 culture medium containing 200 μM acetosyringone. These steps were repeated

three times, with the pellet being resuspended in 400 μ l BY-2 culture medium containing 200 μ M acetosyringone after the final centrifugation step. *Agrobacteria* were then incubated at room temperature for 1 h.

7 ml three-day-old BY-2 suspension culture was used for each transformation. This was mixed with 1.2 μ l 100 mM acetosyringone and 100 μ l of the prepared *Agrobacterium* by gentle inversion before pouring onto solid antibiotic-free BY-2 medium. Plates were sealed with Parafilm and incubated upright in the dark at 27°C.

After 72 h incubation, cells were transferred to sterile 50 ml conical centrifuge tubes. Plates were rinsed into the centrifuge tubes using two 5 ml doses of BY-2 medium containing 100 μ g ml⁻¹ carbenicillin disodium salt and 20 μ g ml⁻¹ Timentin. These antibiotics were added to stop *Agrobacterium* growth. Cells were allowed to settle in the centrifuge tubes. Supernatant was then removed and cells were resuspended in 15 ml BY-2 medium containing carbenicillin disodium salt and timentin. This wash step was repeated a total of three times, with 10.5 ml BY2 medium containing carbenicillin disodium salt and timentin being used for the final resuspension. 1.5 ml of the BY-2 resuspension was plated onto solid BY-2 medium containing 100 μ g ml⁻¹ carbenicillin disodium salt, 20 μ g ml⁻¹ timentin and the appropriate antibiotic to select against untransformed BY-2 cells. Plates were sealed with Parafilm and incubated in the dark at 27°C until the emergence of calli after approximately four to six weeks.

Transgenic calli were subbed to fresh plates containing appropriate selective antibiotic as described in 2.2.1. After growth to a diameter of approximately 2 cm, approximately half of the callus was transferred to 25 ml suspension medium in 50 ml conical flasks and incubated under standard conditions. Cultures were maintained as described in 2.3.1 after they had reached a density approximating seven-day-old wild-type suspension cultures

2.6.13. Transient transformation of tobacco leaves

Agrobacterium was cultured in standard conditions in 5 ml MGL medium (2.5 g l⁻¹ yeast extract, 5 g l⁻¹ tryptone, 5 g l⁻¹ NaCl, 5 g l⁻¹ mannitol, 1.16 g l⁻¹ monosodium glutamate, 0.25 g l⁻¹ KH₂PO₄, 0.1 g l⁻¹ MgSO₄·7H₂O and 1 µg l⁻¹ biotin, pH 7.0). 1 ml of each culture was centrifuged at 2,200g for 5 min. The pellet was resuspended in 1 ml infiltration buffer containing 50 mM 2-(N-morpholino)ethanesulfonic acid (pH 5.6), 2 mM Na₂HPO₄, 0.5% (w/v) glucose and 100 µM acetosyringone. This wash step was repeated twice. OD₆₀₀ was then adjusted with infiltration buffer to approximately 0.1.

Tobacco leaves were dampened with water 5 min prior to infiltration. Holes were made between lateral veins of leaves using a 200 µl pipette tip. 1 ml *Agrobacterium* suspension was injected into the leaf mesophyll using a needleless syringe. Tobacco plants were then grown under standard conditions (25 °C, 16 h light/8 h dark) for 3 days. Infiltrated areas of leaves were harvested, weighed, frozen in liquid nitrogen and lyophilised.

2.7. Determination of isotopic steady state

2.7.1. Incubation of BY-2 cell suspension cultures with 20 mol% [¹³C₆]glucose

Cells were cultured for isotopic steady state determination in 20 ml volumes in 50 ml conical flasks. 2% glucose culture medium contained 20 mol% D-[¹³C₆]glucose (Cambridge Isotope Laboratories, Inc., Hampshire, UK), which contained 99 mol% ¹³C. Cultures were

maintained under standard conditions and harvested by vacuum filtration, as described in 2.3.1. 3 μ l of CCF was dried using a speed-vac and derivatised with MSTFA and MEOX for determination of glucose ^{13}C fractional enrichment by GC-MS.

2.7.2. Extraction of soluble amino acids, organic acids and protein for isotopic steady-state determination

Amino acid extraction

Methanol-soluble metabolites were extracted as described in 2.4.4. Amino acids and organic acids were derivatised with *t*-butyldimethylsilyl (TBDMS) and analysed by GC-MS (Antoniewicz et al., 2007).

Protein extraction from methanol extraction pellets

Protein was extracted from the methanol extraction pellet using 3 ml of 6 M urea and 2 M thiourea, prepared by heating and passing through a 22 μ m syringe filter. Samples were homogenised using a polytron then centrifuged at 20,800g for 30 min at 4°C. The supernatant was centrifuged again. The pellet from each step was retained for starch and cell wall extraction. Protein was precipitated from the supernatant by incubating with 15% (w/v) trichloroacetic acid for 24 h on ice. Samples were then centrifuged for 30 min at 2,800g. The supernatant was discarded and the pellet was washed with ice-cold acetone. Protein was hydrolysed by resuspending the pellet in 2 ml 6 M HCl and heating at 100°C for 24 hours in 15 ml centrifuge tubes the lids of which were studded with 200 μ l pipette tips to create vents. HCl was partially removed by drying using a speed-vac. Samples were then resuspended in H_2O , pH was adjusted to 3-6 using KOH and samples were then centrifuged at 16,000g for

1 min. 5-10 μ l supernatant was dried using a speed-vac and derivatised using TBDMS for GC-MS analysis.

2.7.3. Mass correction of GC-MS data and determination of ^{13}C fractional enrichment

^{13}C fractional enrichment of metabolite fragments recorded by GC-MS was determined after baseline-correction using MetAlign. Elution times of amino acids were determined for several samples simultaneously by searching for ion fragments using MSD ChemStation. This data was supplied to a macro, run in MSD ChemStation, that extracts abundance information for each m/z ratio averaged across the specified time period (Masakapalli, 2011). Mass correction was performed on MATLAB 7.11.0.584 (R2010b, The Mathworks Inc.) using MSCorr (Wahl et al., 2004). This converts absolute m/z abundance values to proportional mass isotopomeric abundances of each metabolite fragment. In addition to this, MSCorr estimates standard deviations of each mass isotopomer proportion based on measurement noise and signal strength. ^{13}C fractional enrichment of each fragment was calculated from this data.

After estimating ^{13}C fractional enrichment, each fragment was validated for reliability based on the estimated ^{13}C fractional enrichment of equivalent fragments from cultures fed unlabelled glucose, and the frequency with which the fragment appeared in the dataset. Fragments appearing in fewer than three replicates or with ^{13}C in unlabelled samples not estimated to be at natural abundance $(0.0113) \pm 0.03$ were ignored. So too were fragments with m/z values that overlapped with other fragments.

2.8. Biomass determination

Biomass composition was determined by following the redistribution of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$, as described by Masakapalli et al. (2013) with appropriate modifications.

2.8.1. Incubation of BY-2 cell suspension cultures with $[\text{U-}^{14}\text{C}]\text{glucose}$

Cultures for use in determining biomass composition were grown under standard conditions until early metabolic steady state. At this point three 5 ml samples were transferred from each culture to autoclaved 100 ml narrow-necked Erlenmeyer conical flasks. To two of these was added 20 μl , 7.4 kBq, D- $[\text{U-}^{14}\text{C}]\text{glucose}$ (10.4 GBq.mmol $^{-1}$, PerkinElmer Inc., Cambridge, UK) in 9% ethanol. The remaining replicate and one of the flasks containing $[\text{U-}^{14}\text{C}]\text{glucose}$ were sealed with a double layer of aluminium foil. The remaining $[\text{U-}^{14}\text{C}]\text{glucose}$ replicate flask was sealed with a rubber bung from which was hung a lidless 0.5 ml microfuge tube and was used for radiorespirometric analysis. Cultures were grown under standard conditions for either 12 h or 24 h.

Cultures in foil-capped flasks were harvested by vacuum filtration. CCF was retained and flasks were rinsed with fresh glucose-free culture medium. Samples were snap-frozen on liquid nitrogen and stored at $-20\text{ }^{\circ}\text{C}$. FW of unlabelled cultures was measured in pre-weighed 15 ml centrifuge tubes. 1 ml CCF was used for scintillation counting.

2.8.2. Radiorespirometric analysis

CO₂ was collected by an alkaline trap method (Harrison and Kruger, 2008) from flasks capped with rubber bungs: CO₂ was released from the medium by addition of 1 ml 12 M formic acid and was collected over 6 h at room temperature in 0.5 ml 10% (w/v) KOH in the suspended microfuge tube. The KOH was transferred to a 5 ml scintillation vial for scintillation counting of ¹⁴CO₂ released by metabolism of [U-¹⁴C]glucose.

2.8.3. Chloroform-methanol extraction of soluble metabolites

Frozen cells were cooled in liquid nitrogen with 10 ml of -20°C methanol. Samples were then thawed on ice for 2 h before being centrifuged for 30 min at 2,800g, 4°C. Supernatant was stored on ice. The insoluble cell residue was resuspended in 4 ml ice-cold methanol, incubated for 5 min on ice and centrifuged as above. The pellet was again suspended in 4 ml methanol, incubated at 70°C for 5 min and centrifuged as above. This step was repeated twice. The pellet was then washed twice more with 4 ml methanol, H₂O was added to the pooled supernatants of each step to double the volume. These and the pellets were then stored at -20°C.

CHCl₃ was added to methanol extracts to create a 1:2:2 (v/v) CHCl₃:CH₃OH:H₂O mixture. This was centrifuged at 3,500g for 10 min at 20°C. The lower CHCl₃ phase (along with precipitated material at the phase interface) was transferred to a fresh centrifuge tube. A further 1:2:2 (v/v) CHCl₃:CH₃OH:H₂O mixture was made with the transferred CHCl₃ phase, and phase-separation was carried out as previously described. This was repeated twice. The aqueous methanol phases were pooled, dried by rotary evaporation at 25°C, and

resuspended in 5 ml H₂O. Radioactivity of 1 ml of the resuspended aqueous methanol extracts and 1 ml of the CH₃Cl extracts from radioisotope-fed cultures was measured by scintillation counting. Corresponding samples from unlabelled cultures were used as blanks. The remaining methanol extract was fractionated by ion-exchange chromatography (IEC).

2.8.4. Ion-exchange chromatography

IEC was performed as described by Kruger et al. (2007)

Preparation of resins

Anion exchange resin (Dowex-1, 1*8-200 (Sigma-Aldrich Co. LLC Poole, UK)) was washed three times in excess H₂O by allowing to the resin to settle and removing the unsettled finings. It was charged by washing it three times in excess 1 M sodium acetate, followed by 0.1 M acetic acid until the suspension became acidic.

Cation exchange resin (Dowex-50, 50*8-200 (Sigma-Aldrich Co. LLC Poole, UK)) was incubated at 100°C for 1 h in two volumes of 4 M HCl. It was then washed in a Büchner funnel with H₂O until the pH of the flow-through became neutral. The resin was then heated at 100°C with two volumes of 10% (w/v) NaOH for 1 h. It was washed again with H₂O until the flow-through became neutral and stored in two volumes of 2 M HCl at 4°C. The resin was charged by washing three times in excess 2 M HCl and then washing with H₂O until the pH was stable.

Sample fractionation

Ion-exchange columns were created by stopping two 1 ml pipette tips with approximately 100 μ l glass beads and then adding 500 μ l of either the charged anion exchange resin or the charged cation exchange resin. Excess liquid was drained from each column by exerting slight positive pressure on the top of the column. The cationic column was then placed on top of the anionic column and the assembly was suspended above an appropriate collecting vessel.

Sample pH was adjusted to 3-6 using 0.1 M KOH or HCl prior to fractionation. Sample was applied to the column assembly in 1 ml volumes and passed through under slight positive pressure. Columns were then washed three times with 0.8 ml H₂O. The flow-through (neutral fraction, containing sugars) was retained. Columns were then separated. The basic fraction (containing amino acids) was eluted from the cation exchange column with 2 ml 1 M NH₄OH. The acidic fraction (containing organic acids) was eluted from the anion exchange column with 2 ml 2 M formic acid.

IEC Fractions were lyophilised and then resuspended in either 1 ml (acidic and basic fractions) or 2 ml (neutral fraction) H₂O. Radioactivity was measured in 1 ml of resuspended samples from ¹⁴C samples by scintillation counting. 12 μ g ribitol was added as an internal standard to unlabelled samples. 60 μ l amino acid, 10 μ l neutral, 80 μ l organic acid samples were dried by speed-vac, derivatised using MSTFA and MEOX, and analysed by GC-MS as described in 2.2.1. 10 μ l of alkane mix was added with MSTFA to allow analysis using TagFinder, as described in 2.4.4.

2.8.5. Further separation of sugars by thin layer chromatography

The remaining 1 ml neutral IEC fraction was lyophilised and resuspended in 50 µl of 50% (v/v) ethanol. 25 µl was spotted onto 20 cm x 20 cm Polygram CEL 300 0.1 mm cellulose TLC plates (Macherey-Nagel GmbH & Co., Germany) and developed three times in a solvent of 15% (v/v) formic acid, 30% (v/v) butanone, 40% (v/v) *tert*-butanol. TLC plates were analysed by phosphorimaging for 72 h. 25 µl 50% (v/v) ethanol was used as a blank.

2.8.6. Extraction of starch, protein and cell wall from methanol extraction pellets

The insoluble pellet resulting from methanol extraction of radioisotope-fed cultures was resuspended to 2 ml with H₂O. Radioactivity of a 100 µl aliquot was measured after incubating at 60 °C for 16 h with 400 µl Solvable tissue solubiliser (PerkinElmer Inc., Cambridge, UK). The remaining 1.9 ml was processed as described by Malone et al. (2006). Samples were autoclaved for 3 h at 121 °C and then incubated for 16 h at 37 °C with 400 µl 200 mM sodium acetate (pH 4.8) containing 10 U of amyloglucosidase (from *Aspergillus niger*, Roche Diagnostics Ltd, Burgess Hill, UK) and 20 U of α-amylase (from *Bacillus amyloliquefaciens*, Roche Diagnostics Ltd, Burgess Hill, UK). 400 µl 400 mM Tris-HCl (pH 7.8) containing protease XIV (from *Streptomyces griseus*, Sigma-Aldrich Co. LLC Poole, UK) was added, and samples were incubated for a further 16 h at 37 °C.

Undigested residue was pelleted by centrifuging at 2,800g for 30 min and rinsed with 2 ml H₂O. Supernatants were separated by IEC into starch (neutral), cell wall (acidic) and protein

(basic) fractions. Dried IEC fractions were resuspended to 1 ml and scintillation counted. Pellets were solubilised at 60 °C for five days with Solvable tissue solubiliser in 2 ml volumes. H₂O was then added to solubilised pellets to a volume of 10 ml. 1 ml was used for scintillation counting.

2.8.7. Extraction of ethanol, organic acids and protein from cell culture filtrate

Radioactive ethanol was distilled from 5 ml CCF using 2 ml ethanol twice as a carrier. 1 ml of the distillate was used for scintillation counting. The remaining 3 ml distillate was allowed to dry, resuspended in 1 ml H₂O and scintillation counted to allow subtraction of the radioactivity of any other metabolites that carried over with the ethanol from the radioactivity of the ethanol.

Excreted organic acids and were fractionated from the CCF by IEC using the anion exchange column only. 250 µl of resuspended organic acid fraction from unlabelled samples was used for GC-MS. 5 µl of 0.2 mg ml⁻¹ norvaline in 0.1 M HCl was added as an internal standard to allow quantification of citrate and isocitrate. Samples were dried by speed-vac and derivatised with MSTFA and MEOX.

A 2:15 (w/v) solution of sodium deoxycholate in 100% (w/v) trichloroacetic acid was prepared by heating and thorough mixing using a vortex. Protein was extracted from CCF of ¹⁴C samples by adding 205 µl ml⁻¹ of this solution and 2 mg ml⁻¹ BSA to the CCF and incubating on ice overnight. Samples were then centrifuged at 13,000g for 15 min. The supernatant was retained for scintillation counting. The protein pellet was washed with two

volumes of 1 ml acetone and centrifuged for 15 min at 13,000*g*. Radioactivity of the pellet was measured after resuspending in 1 ml H₂O.

2.9. Metabolic flux analysis methods

2.9.1. Incubation of BY-2 cell suspension cultures with [¹³C]glucose

Cells were cultured for metabolic flux analysis (MFA) in 20 ml volumes in 50 ml conical flasks. [1-¹³C]glucose (Cambridge Isotope Laboratories, Inc., Hampshire, UK), which had 99 mol% glucose labelled with ¹³C at the 1-C position, and 20 mol% [¹³C₆]glucose was used in parallel labelling experiments during MFA.

Cultures were maintained under standard conditions, harvested by vacuum filtration and rinsed with two 10 ml volumes of glucose-free medium. 3 µl of the filtrate was dried using a speed-vac and derivatised with MSTFA and MEOX for GC-MS analysis of the ¹³C fractional enrichment of the supplied glucose.

2.9.2. Extraction of soluble amino acid, protein and starch for isotopic steady-state determination and metabolic flux analysis

Extraction of soluble amino acid and protein

Methanol-soluble metabolites were extracted as described in 2.4.4 and derivatised with TBDMS for GC-MS analysis. Protein was extracted from methanol extraction pellets and analysed by GC-MS as described in 2.7.2.

Starch extraction from methanol extraction pellets

Starch was extracted from protein-free pellets. Urea and thiourea were removed by washing twice with 1.5 ml H₂O and centrifuging for 30 min at 19,000g, 4°C. Pellets were resuspended in 5 ml H₂O and autoclaved at 121°C for 3 h in 15 ml centrifuge tubes the lids of which were studded with 200 µl pipette tips to create vents. H₂O was removed by transferring the autoclaved suspension to microfuge tubes and centrifuging at 19,000 for 30 min. The pellet was then resuspended in 3 ml 200 mM sodium acetate (pH 4.8) containing 20 U α-amylase and 10 U amyloglucosidase and incubated at 37°C overnight. Samples were then centrifuged 2,800g for 15 min. 7-10 µl of supernatant containing glucose derived from starch was dried by speed-vac and derivatised with MSTFA and MEOX for GC-MS analysis. The pellet was retained for cell wall hydrolysis.

Cell wall extraction from methanol extraction pellets

Cell wall material was extracted from protein-free and starch-free pellets by heating at 120°C for 1 h in 2 M trifluoroacetic acid. Samples were then centrifuged at 2,800g for 12 min. Supernatant containing hydrolysed cell wall was centrifuged again at 16,000g for 10 min to remove any remaining insoluble residue. 10 µl of the supernatant was then dried by speed-

vac, resuspended in 20 µl methanol, and dried again to remove traces of trifluoroacetic acid. Dried samples were then derivatised with MSTFA and MEOX for GC-MS analysis.

2.9.3. Metabolic modelling using 13C-FLUX

GC-MS data was mass corrected and mass fragments were validated as described in 2.7.3. Steady-state MFA modelling was performed using 13C-FLUX (version 20050329; Wiechert et al., 2001) on Ubuntu 10.04 (Canonical Ltd, London, UK). A published model for a heterotrophic *Arabidopsis thaliana* (L.) Heynh. (ecotype Landsberg erecta) cell suspension culture (Masakapalli et al., 2010) was used as a template.

Specifying output fluxes

Output fluxes, determined from redistribution of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$ were initially defined with their standard deviations in the “flux measurements” section of each model and were set as free (F) in the “fluxes” section. These were later constrained to the best fitting value that was within one standard deviation of the experimentally determined value.

Correcting for unlabelled pools of metabolites

Proxy reactions were included to account for unlabelled pools of arginine, glycine, histidine, methionine and serine, which have carbon contributions from exogenous (unlabelled) CO_2 or methyl-tetrahydrofolate. Other protein-derived amino acids, and sugars derived from starch and cell wall were corrected for pre-existing unlabelled pools using a mathematical procedure based on the labelling of primary metabolite precursors that were at near isotopic steady state (Masakapalli et al., 2013). This was done by deriving a correction factor (Δ) for each mass isotopomer, defined as:

$$\Delta = \frac{F_S - F_P}{F_S - 0.0113}$$

where F_S is the expected ^{13}C fractional enrichment based on the fractional enrichment in precursor metabolites, F_P is the measured fractional enrichment in that fragment, and 0.0113 is the ^{13}C natural abundance. The product of Δ and the expected natural abundance of the mass isotopomer was subtracted from the normalised abundance of the mass isotopomer. This value was then normalised to the sum of the corrected mass isotopomers for that metabolite fragment.

Modelling metabolic fluxes

Modelling using 13C-FLUX was an iterative procedure that involved:

1. Validating the model syntax by using the command *Ftbl2Flx* “Model”.*ftbl*.
2. Simulating the model using the command *CumoNet* “Model”.*ftbl* -a 0.00001 > *CumoNet_out.txt* to determine if the initial flux values and categorisation of fluxes (whether initially specified by the user and adjusted by the software (F), constrained by the user (C) or determined by the model based on the values of other fluxes (D)) resulted in a feasible solution.
3. Minimising the sum of squared differences (residuum) between the experimental measurements and those predicted by the model, when free flux values are altered, by using the command *Donlp2* “Model”.*ftbl* -a 0.00001 -m 0 -bootstrap -logAllF > *DON_OUT.txt* (P. Spelluci, Technische Universität Darmstadt, Germany).

At this point the contribution of different measurements to the residuum was checked in the output file *out.residuumscanner.txt*. The validity of measurements contributing significantly to the residuum was manually assessed on the basis of the degree of agreement between fragments in the same sample that report on the same carbon atoms, and on the abundance of the

fragment as recorded by the GC-MS. Fluxes were remodelled after omission of unreliable data.

A global best-fit model was determined by performing Monte Carlo simulations with bootstrapping of the mass isotopomer measurements using *Donlp2 "Model".ftbl -a 0.00001 -m N -bootstrap -logAllF > DON_OUT.txt* command, where N is the number of Monte Carlo simulations, then invoking *Donlp2 "Model".ftbl -a 0.00001 -m 0 -bootstrap -logAllF > DON_OUT.txt* a final time with initial free flux values set as the average values of the Monte Carlo simulated results with low residua.

2.9.4. Statistical analysis of metabolic flux analysis results

Linear statistical analysis of free and model-determined fluxes was performed by invoking *EstimateStat "Model".ftbl -a 0.00001 > EstimateStat_out.txt*. 68% confidence intervals were determined by taking the range of the middle 68% of values for each flux from Monte Carlo simulated solutions with low residua. 95% confidence intervals were determined by varying each free flux in turn and measuring its effect on the best-fit residuum (Antoniewicz et al., 2006).

Chapter 3. Metabolic analysis of wild-type BY-2 cells and validation of experimental methods

3.1. Introduction

3.1.1. Objectives

The aims of this chapter were to verify that tobacco (*Nicotiana tabacum*) Bright Yellow 2 (BY-2) cells are an appropriate platform for metabolic flux analysis (MFA) and to validate that the analytical methods used in later chapters can be performed accurately and reliably on BY-2 cultures and BY-2 cell extracts.

3.1.2. Cell suspension cultures are useful for metabolic engineering and metabolic flux analysis

Cell suspension cultures are particularly apt for the study of metabolism due to their high growth rate and relative uniformity of cell type, which removes the difficulties associated with multiple cell-types and makes for easier sample processing and result analysis. Cell suspension cultures can also be grown heterotrophically, allowing for MFA to be performed (Kruger et al., 2007; Matsuda et al., 2007; Williams et al., 2008; Masakapalli et al., 2010).

Cell suspension cultures are used for commercial biologics synthesis, with the most notable financially viable example being the production of paclitaxel by *Taxus spp.* (Roberts and Shuler, 1997). There are also numerous other examples of metabolic engineering of plant cell

suspension cultures in an academic context (Gondet et al., 1992; Hefner et al., 1998; Koyama et al., 1999; Goossens et al., 2003; Inui et al., 2007).

As cell suspension cultures can be grown quickly to a large biomass in standard bioreactors, and product purification costs are generally lower than from whole plants (Xu et al., 2011a), they are particularly suited for the industrial production of high value products that are naturally produced by slow-growing and often ecologically vulnerable species. Also, unlike most crop plants, cell suspension culture growth in bioreactors can be climate-independent, allowing year-round growth. Cell suspension cultures grown in sterile bioreactors are protected from pathogens and can also overcome containment worries surrounding transgenic crops, particularly when scale-up is desired. Plant cell suspension cultures additionally have some advantages over bacterial cell cultures, including the possession of a wider repertoire of post-translational modification and protein folding strategies, which can be particularly relevant if the desired product is a eukaryotic protein or the product of a eukaryotic enzyme. However, high culture maintenance costs (Xu et al., 2011a), genetic instability (Weathers et al., 2010) and karyotype diversity (Kovarík et al., 2012) have prevented their widespread commercial use.

3.1.3. Tobacco BY-2 cells are the optimal cell suspension culture for studying sterol biosynthesis

Tobacco BY-2 cells were initiated from an induced callus of *Nicotiana tabacum* L. “Bright Yellow 2” in Japan more than four decades ago (Kato et al., 1972). They are relatively easily and stably transformed (David and Perrot-Rechenmann, 2001), have a high growth rate (even for cell cultures) (Nagata et al., 1992) and are obligate heterotrophs (Hemmerlin et al., 2004).

They also have proven potential for industrial biotechnology, particularly for the production of recombinant therapeutic proteins (Lienard et al., 2007; Sack et al., 2007; Xu et al., 2007; Kaldis et al., 2013).

More pertinently, BY-2 cells have high 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity, even compared to sterol over-producing strains of other tobacco lines (Gondet et al., 1992), and this translates to high sterol levels (Hemmerlin and Bach, 2000; Wentzinger et al., 2002; Hemmerlin et al., 2004). They are an excellent model organism for studying sterol biosynthesis, as evidenced by a rich body of research into the regulation of sterol metabolism in BY-2 cells (Disch et al., 1998; Hemmerlin and Bach, 1998; Hemmerlin and Bach, 2000; Hartmann and Bach, 2001; Wentzinger et al., 2002; Hemmerlin et al., 2003; Hemmerlin et al., 2004; Hemmerlin et al., 2006a; Hemmerlin et al., 2006b; Bonneau et al., 2010).

3.2. Results

3.2.1. Growth analysis of BY-2 cultures

Growth analysis was performed to determine a medium that would be suitable for culture maintenance and experimental work. Growth analysis also provides a useful indication of culture health, and knowledge of the timing of logarithmic growth is required for MFA, enzyme assays and transformations.

BY-2 growth in 3% sucrose, 2% glucose and 3% glucose medium

Steady state MFA involves following redistribution of isotopic labels supplied by specifically labelled substrates. BY-2 cells are conventionally cultured with sucrose as a carbon source (Brandizzi et al., 2003). However, isotopically labelled sucrose is expensive compared to isotopically labelled glucose. As such, a suitable glucose medium for BY-2 cultures initially grown in 3% (w/v) sucrose medium was desirable. Medium containing 2% glucose has previously been determined to be optimal for BY-2 cultures (Disch et al., 1998), so growth was analysed for cultures grown in medium containing either 2% or 3% glucose. The latter was chosen to maintain the concentration of hexose units in 3% sucrose medium. Cells were allowed to acclimate to the change in culture medium for five subculture cycles before 24-hourly measurement of fresh weight (FW).

The highest FW was attained in 3% sucrose medium (Figure 3.1a), in which a 2-day lag phase was followed by a rapid increase in FW until four days after subculture, at which point growth slowed and became stationary by seven days at $447 \pm 13 \text{ mg ml}^{-1}$ FW (mean $\pm$ standard error (SEM), $n = 5$). Evans blue staining of dead cells indicated that $95.4\% \pm 1.0\%$ (mean $\pm$ SEM, $n = 4$) of cells were viable at eight days. 2% glucose cultures had an extended log phase duration, until five days. 3% glucose cultures also exhibited a log phase from two to five days, although growth was slower than in 2% glucose. 2% and 3% glucose cultures achieved final masses of $78.1\% \pm 1.3\%$ and $68.4\% \pm 0.6\%$ (mean $\pm$ SEM, $n \geq 4$), respectively, of the total FW of 3% sucrose cultures. Therefore, subsequent experiments used 2% glucose cultures.

Comparisons of dry weight and fresh weight

2% glucose culture samples used for FW growth analysis were freeze-dried and dry weight (DW) was measured (Figure 3.1b). The DW profile corresponded broadly with the FW profile, with DW equating to $3.6\% \pm 0.6\%$ of FW (mean $\pm$ SEM). However, in cultures older than

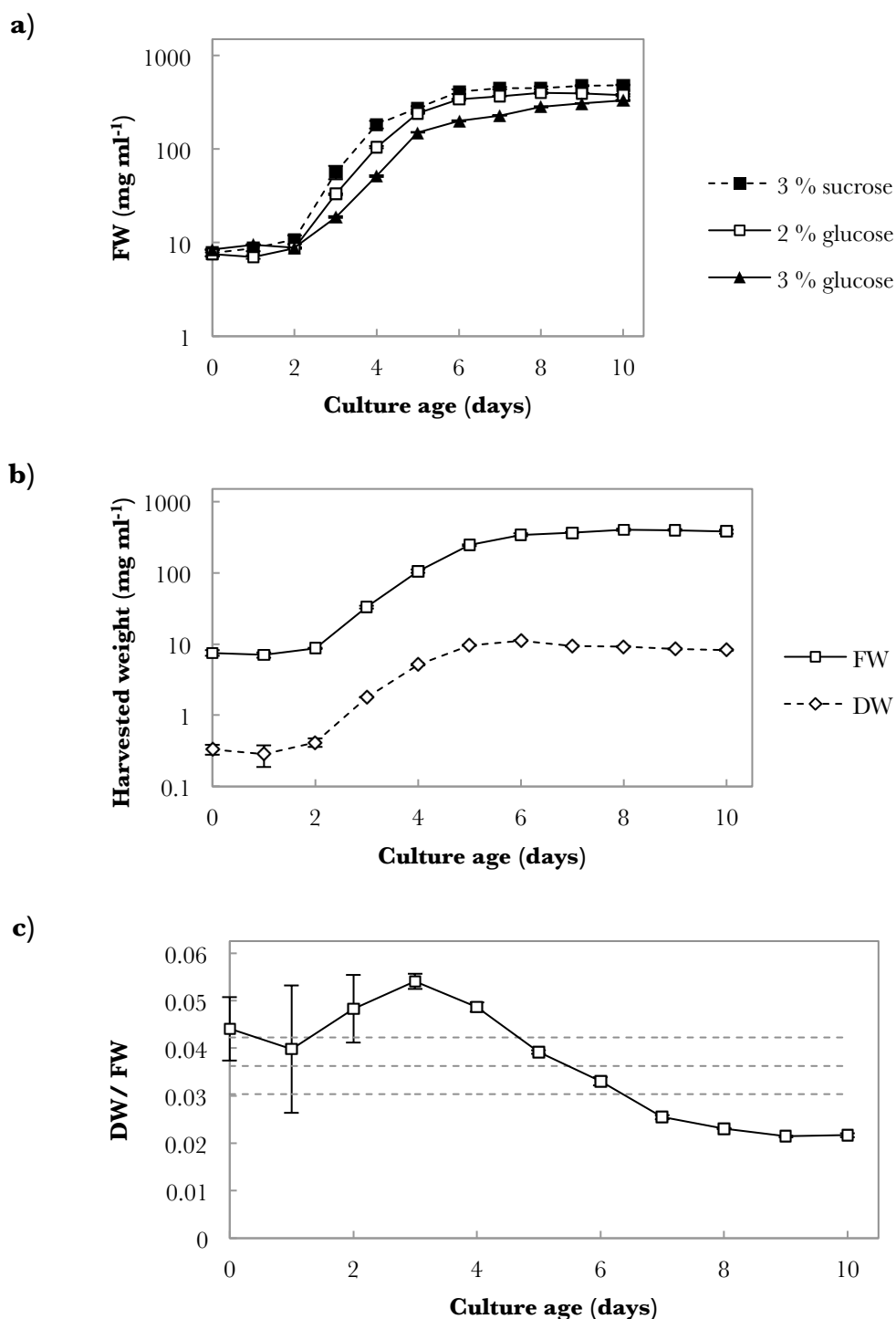


Figure 3.1. Growth analysis of wild-type BY-2 cells in suspension culture.

Wild-type (WT) BY-2 was cultured in media containing 3% (w/v) sucrose, 2% glucose or 3% glucose. **(a)** FW was measured at 24 h intervals, beginning with the time of subculture, to allow comparison of growth between the media. **(b)** 2% glucose samples were then dried by lyophilisation and reanalysed. **(c)** This allowed direct comparisons of DW and FW. Data points represent mean $\pm$ SEM, $n \geq 4$. Dotted lines in **(c)** represent overall mean $\pm$ standard error.

three days, the observed DW to FW ratio dropped steadily from $5.4\% \pm 0.2\%$ to a low of $2.1\% \pm 0.0\%$ by nine days (Figure 3.1c). This resulted in a less defined transition from logarithmic to stationary growth when DW was assessed. Also, the DW to FW ratio was highly variable in cultures less than three days (prior to log-phase).

In an attempt to determine the reason for the observed drop in the DW to FW ratio, entire volumes of replicate cultures were harvested by vacuum filtration at 24 h intervals, and DW and FW were measured. The DW to FW ratio dropped from 6% at two days to 4% at 10 days. Measurements from cultures younger than two days were again highly variable. Similar experiments on transgenic lines had similar results.

3.2.2. Optimisation of metabolite assays and analysis of wild-type BY-2 cell cultures

3.2.2.1. Sterols

Unlike animals and fungi, which produce a single major sterol, plants synthesise a variety of different sterols. More than 60 sterols have been identified in maize alone (Guo et al., 1995), although plants tend to have a core group of three or four sterols that make up the majority of their sterol profile. In BY-2 cells, campesterol, sitosterol and stigmasterol are the most abundant sterols, together accounting for more than 75% of the plasma membrane sterol content (Wentzinger et al., 2002; Mongrand et al., 2004; Roche et al., 2008).

Identification and quantification of individual sterols was required for determination of the effects of genetic and chemical manipulations on sterol biosynthesis. The optimum means of

sterol identification was determined initially by analysing the commercially available Matreya plant sterol mix (Table 3.1) and BY-2 hexane extracts using gas chromatography-mass spectrometry (GC-MS) and ^1H nuclear magnetic resonance (NMR) spectroscopy.

Identification of sterols by GC-MS

GC-MS of the Matreya plant sterol mix resulted in GC-MS chromatograms with well-defined peaks that matched the profile on the supplied plant sterol mix data sheet. Searches of a GC-MS library of methoxyamine (MEOX) and trimethylsilyl (TMS) plant metabolite derivatives (Kopka et al., 2005) led to the positive identification of campesterol, stigmasterol and sitosterol. These identities were verified, along with brassicasterol (which was not in the GC-MS library), by comparison of the experimental gas chromatogram with the Matreya plant sterol mix data sheet gas chromatogram.

Several sterols in hexane extracts from four-day-old and seven-day-old BY-2 cultures were identified by comparison with the GC-MS library (cholesterol, campesterol, stigmasterol, sitosterol, fucosterol and cycloartenol), whereas literature searches led to the identification of 24-methylene cholesterol (Weete and Gandhi, 1997) and Δ^5 -avenasterol, the C-24,24¹ stereoisomer of fucosterol (Kamal-Eldin et al., 1998; Xu et al., 2012) (Figure 3.2; Appendix 3.1). No sterols were detected in negative control samples, which were created using the extraction and derivatisation procedures without dried cells.

Identification of sterols by ^1H -NMR spectroscopy

A literature search of sterol ^1H -NMR spectroscopy data revealed a lack of agreement on chemical shift values (Table 3.2). Chemical shifts from the Matreya plant sterol mix were compared with the chemical shifts from the literature in an attempt to positively identify sterols in the mixture. However, some values for each of the expected sterols were not seen in

Table 3.1. Composition of the Matreya plant sterol mix.

Compounds are ordered by GC elution time.

Analyte	Molar Proportion	Stock concentration
Brassicasterol	0.13	3.25 mg ml ⁻¹
Campesterol	0.26	6.5 mg ml ⁻¹
Stigmasterol	0.07	1.75 mg ml ⁻¹
Sitosterol	0.53	13.25 mg ml ⁻¹
Total	1	25 mg ml⁻¹

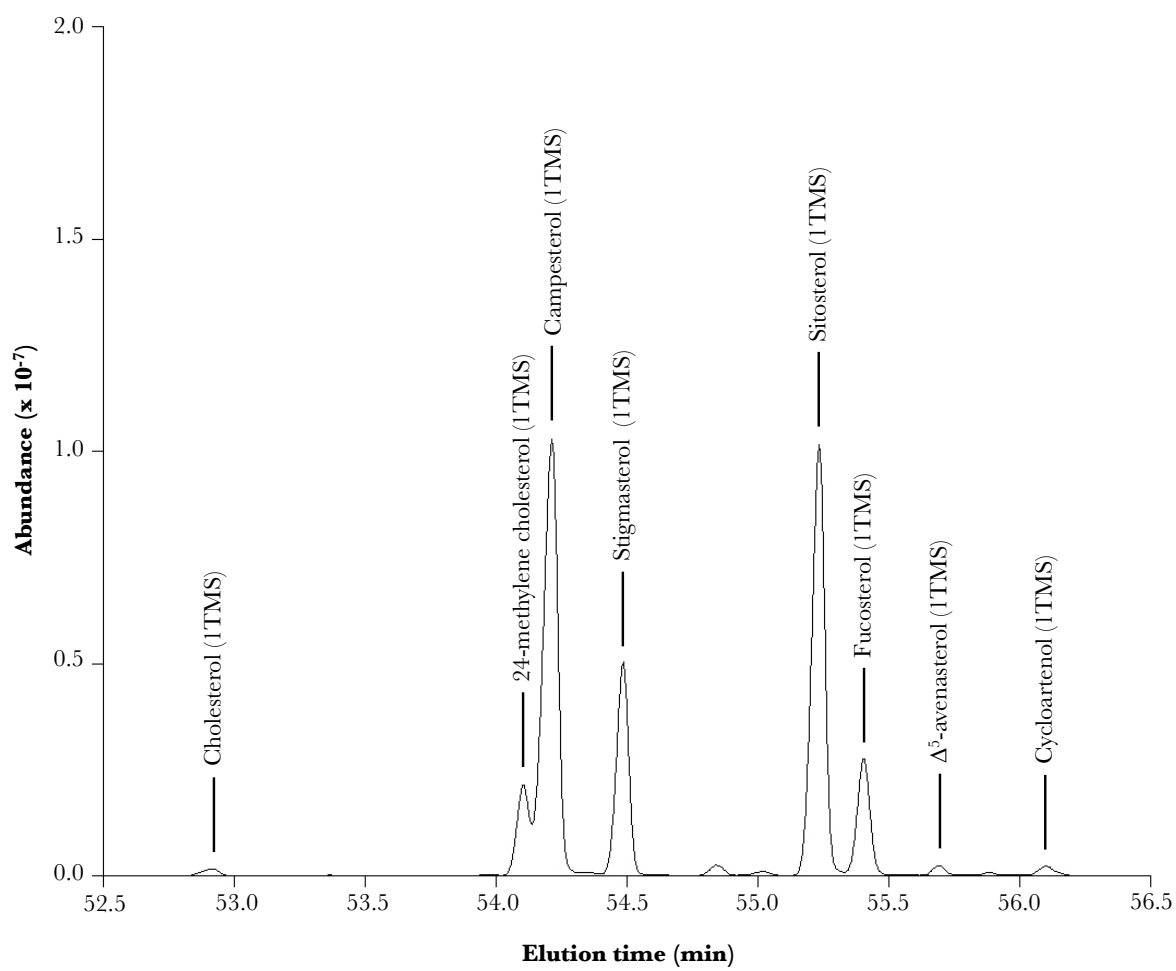


Figure 3.2. GC-MS chromatogram of a hexane extract from a four-day-old wild-type BY-2 culture (52.5 to 56.5 min).

Sterols were identified by comparison of elution times and mass spectra with a library of compounds and values from published literature.

Table 3.2. Published sterol ¹H-NMR spectroscopy chemical shifts and their presence in the Matreya plant sterol mix.

Presented are details of reference chemical shifts for the four sterols in the plant sterol mix in CDCl₃. Details for cholesterol and 24-methylenesterol are presented as outliers. It should be noted that some H positions are not accounted for in some of the references. Chemical shifts that are unique to a particular sterol are underlined. Chemical shifts identified in the Matreya plant sterol mix are in **bold**. Corresponding C positions are in brackets.

Analyte	Chemical shift (PPM)	References
Brassicasterol	5.35-5.14 (22), 5.32 (6), 5.16 (23), 3.51-3.49 (3), 1.24-0.89 (21), 1.02-0.99 (19), 0.98-0.82 (27), 0.92-0.81 (24 ¹), 0.85-0.79 (26), 0.71-0.67 (18)	(Rubinstein et al., 1976; Iida et al., 1980; Matsumoto et al., 1983; Hayee-Memon et al., 1991; Lee et al., 2010)
Campesterol	5.36-5.10 (6), 3.53-3.21 (3), 2.27-2.21 (4), 2.04-1.94 (7, 12), 1.88-1.79 (1, 2, 16), 1.62-1.02 (5, 1, 17, 12, 23, 24, 16, 20, 22, 25, 8, 11, 2, 15), 1.03-0.96 (19), 0.96 (19), 0.96-0.91 (21), 0.86-0.85 (26), 0.85 (24 ²), 0.81-0.80 (27), 0.81-0.77 (24 ¹), 0.79-0.77 (24), 0.70-0.68 (18)	(Rubinstein et al., 1976; Iida et al., 1980; Kalinowska et al., 1990; Guo et al., 1995; Zhang et al., 2006; Wishart et al., 2009; Jain and Bari, 2010)
Sitosterol	5.56-5.23 (6), 3.52-3.32 (3), 2.29-1.08 (4, 25), 1.03-0.93 (19), 0.92-0.85 (21), 0.88-0.77 (24 ²), 0.85-0.74 (26), 0.82-0.72 (27), 0.69-0.61 (18)	(Rubinstein et al., 1976; Iida et al., 1980; Kalinowska et al., 1990; Kojima et al., 1990; Guo et al., 1995; Shameel et al., 1996; Chung et al., 2005; Zhang et al., 2006; Lyu et al., 2009; Wishart et al., 2009; Lee et al., 2010)
Stigmasterol	5.35-5.14 (6), <u>5.17-4.16</u> (22), <u>5.15-4.14</u> (23), 3.62-3.21 (3), 2.30-2.23 (4), 2.06-2.02 (20), 2.02-1.97 (7 α), 2.02-1.83 (2), 2.00 (12), 1.86-1.84 (1), 1.72-1.71 (16), 1.56-1.47 (15), 1.55-1.47 (25), 1.54-1.17 (24), 1.51 (2), 1.50-1.47 (7), 1.47-1.46 (8), 1.47-1.43 (24 ¹), 1.28-1.17 (16), 1.27-0.90 (21), 1.19-0.80 (19), 1.18-1.17 (12), 1.18 (24 ¹), 1.17-1.15 (17), 1.17-1.08 (1), 1.17-1.06 (15), 1.07-0.82 (26), 1.02-1.01 (14), 1.01-0.94 (9), 1.00-0.79 (24 ²), 0.98-0.76 (27), 0.91-0.65 (18)	(Iida et al., 1980; Kojima et al., 1990; Guo et al., 1995; Shameel et al., 1996; Forgo and Kövér, 2004; Lyu et al., 2009; Wishart et al., 2009; Jain and Bari, 2010)
Cholesterol	5.35-5.32 (6), 3.52-3.48 (3), 2.27-2.20 (4), 2.20-1.96 (7, 12), 1.87-1.52 (7, 15, 25), 1.82-1.11 (1, 2, 12, 15, 16, 17, 20, 22, 23, 24), 1.37-0.97 (9, 14, 22), 1.02-0.99 (19), 0.92-0.86 (26), 0.92-0.85 (27), 0.92-0.84 (21), 0.70-0.68 (18)	(Iida et al., 1980; Kalinowska et al., 1990; Guo et al., 1995; Ravichandran and Divakar, 1998; Wishart et al., 2009)
24-methylene-cholesterol	<u>5.72</u> (5), 5.44-5.36 (6), <u>4.69-3.34</u> (24 ¹), 3.53-3.52 (3), 1.57-1.03 (26, 27), 1.24-0.91 (21), 1.02-0.87 (19), 0.70-0.65 (18)	(Iida et al., 1980; Kalinowska et al., 1990; Hayee-Memon et al., 1991; Guo et al., 1995; Lo et al., 2001)

the spectra, and several peaks for unexpected sterols (e.g. cholesterol and 24-methylene cholesterol) were evident. This was due to there being many chemical shift values shared between different sterols. Only in the case of sitosterol was a chemical shift value detected in the Matreya plant sterol mix spectra that is not recorded in the literature for one of the other five researched sterols. Analysis was further complicated by low resolution of the NMR peaks, meaning that it was not possible to determine peak type (whether singlet, doublet, triplet, etc.).

The presence of fatty acids (and possibly smaller compounds too volatile to be analysed by GC-MS) made identification of sterols in BY-2 hexane extracts by ¹H-NMR even less feasible than in the Matreya plant sterol mix (Figure 3.3). There was no single chemical shift value that could be assigned to only one of the six researched sterols (Table 3.3).

Quantification of sterols by GC-MS

Nonadecanoic acid (NDA) was used as an internal standard for the quantification of sterols, in order to overcome variability in the GC-MS detector response. Comparisons of integrated sterol peaks with integrated NDA peaks were made over injected sterol concentrations ranging from 1 to 1210 ng µl⁻¹ using the Matreya plant sterol mix. The response was linear up to 405 ng µl⁻¹ and was similar for each sterol (Figure 3.4). Measurements of peak heights (sterol/NDA) were only linear up to 66 ng µl⁻¹, and the response values for each concentration were more broadly spread than when using integrated peaks (Appendix 3.2). Linear regression of the integration data for concentrations less than 405 ng µl⁻¹ ranged from $y = 0.0123x$ to $y = 0.0138x$ compared to $y = 0.0130x$ to $y = 0.0154x$ for peak height data. Linear regression of pooled peak area data gave a single quantification formula for sterols:

$$Sterol_{ng,\mu l^{-1}} = 79.87 \times \left(\frac{Sterol_{area}}{NDA_{area}} \right)$$

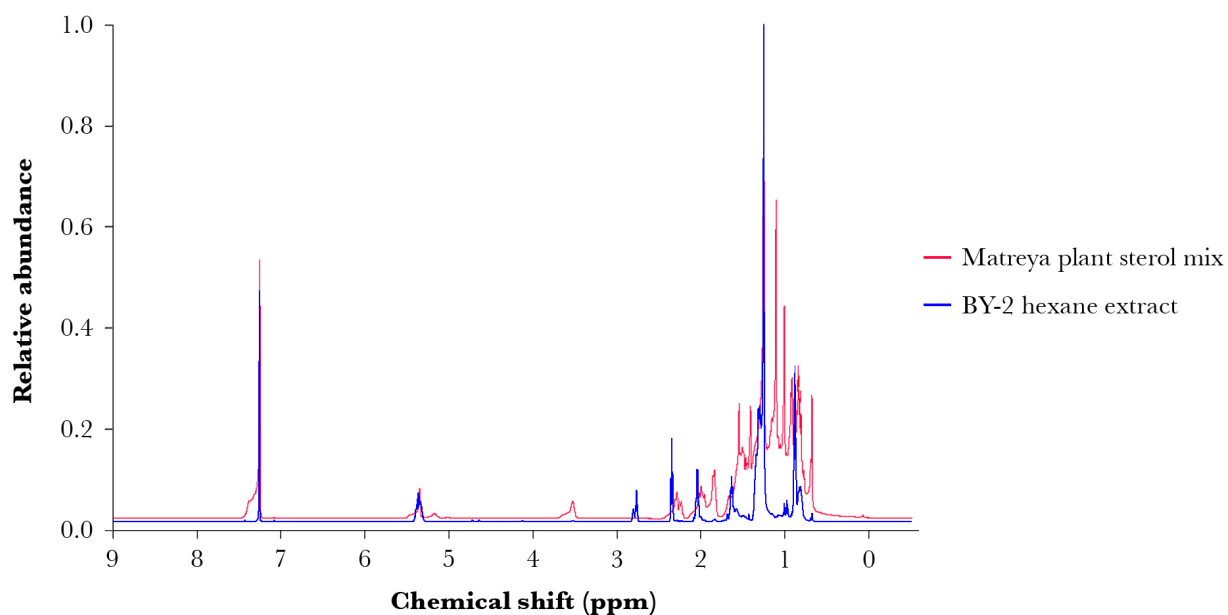


Figure 3.3. ¹H-NMR spectra of the plant sterol mix and a wild-type BY-2 hexane extract.

The ¹H-NMR spectrum of a BY-2 hexane extract overlays that of 50 µl Matreya plant sterol mix. There are shared peaks at 7.26 ppm (CHCl₃) and 5.36 ppm, but the region at less than 4 ppm differs between the two samples, probably due to a lower concentration of sterols in the hexane extract and a higher concentration of other H-containing compounds.

Table 3.3. ¹H-NMR chemical shifts of BY-2 hexane extract.

These are the chemical shift values of BY-2 hexane extracts along with possible sterols that they could represent. Spectra were not of sufficient resolution to determine whether chemical shifts were singlets, doublets or multiplets.

Chemical shift (ppm)	Possible identity
5.38	Sitosterol, 24-methylenecholesterol
5.36	Campesterol, sitosterol, 24-methylenecholesterol
5.35-5.34	Brassicasterol, campesterol, sitosterol, stigmasterol, cholesterol
2.78-2.76	Unknown
2.36-2.34	Unknown
2.07	Sitosterol, cholesterol
2.05	Sitosterol, stigmasterol, cholesterol
2.04-2.03	Campesterol, sitosterol, stigmasterol, cholesterol
1.65	Sitosterol, cholesterol
1.63	Sitosterol, cholesterol
1.62-1.61	Campesterol, sitosterol, cholesterol
1.35-1.34	Campesterol, sitosterol, stigmasterol, cholesterol, 24-methylenecholesterol
1.32-1.29	Campesterol, sitosterol, stigmasterol, cholesterol, 24-methylenecholesterol
1.25	Campesterol, sitosterol, stigmasterol, cholesterol, 24-methylenecholesterol
1.01	Brassicasterol, campesterol, sitosterol, stigmasterol, cholesterol, 24-methylenecholesterol
0.98	Brassicasterol, campesterol, sitosterol, stigmasterol, cholesterol, 24-methylenecholesterol
0.90-0.87	Brassicasterol, sitosterol, stigmasterol, cholesterol, 24-methylenecholesterol
0.85	Brassicasterol, campesterol, sitosterol, stigmasterol, cholesterol
0.84	Brassicasterol, sitosterol, stigmasterol, cholesterol
0.83-0.82	Brassicasterol, sitosterol, stigmasterol
0.81	Brassicasterol, campesterol, sitosterol, stigmasterol

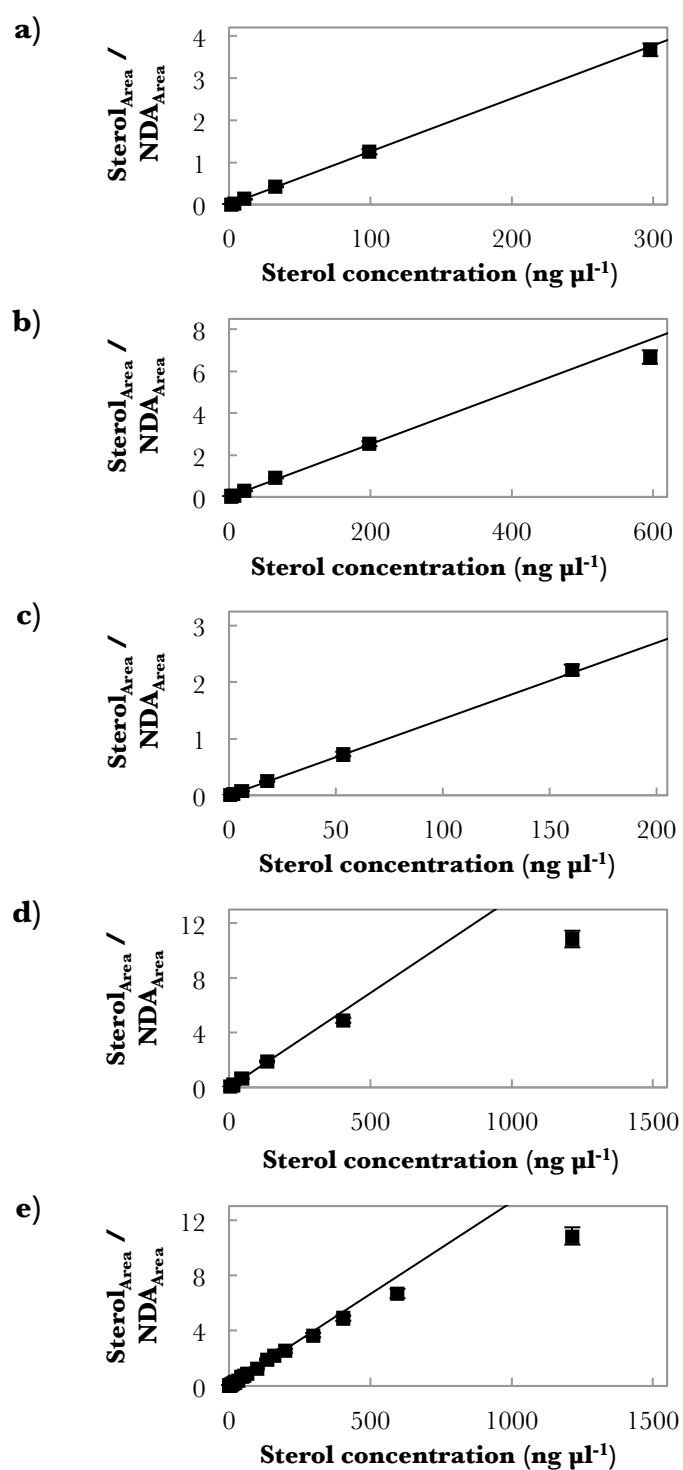


Figure 3.4. Calibration curves of sterols quantified by GC-MS peak area.

Linear regression formulae for injected concentrations up to 405 ng µl⁻¹ for 1TMS sterol esters were **(a)** $y = 0.0123x$ ($R^2 = 0.990$) for brassicasterol, **(b)** $y = 0.0129x$ ($R^2 = 0.985$) for campesterol, **(c)** $y = 0.0138x$ ($R^2 = 0.989$) for stigmasterol, and **(d)** $y = 0.0123x$ ($R^2 = 0.985$) for sitosterol. **(e)** The linear regression formula for all sterols was $y = 0.0125x$ ($R^2 = 0.986$). Values = mean $\pm$ SEM, $n = 5$. R^2 values are for all replicates.

Recovery of sterols by alkaline saponification and hexane extraction

The reliability of total sterol extraction and the proportion of sterols recovered was determined by spiking 50 mg dried BY-2 cells with 5 μ l Matreya plant sterol mix (containing 16.3 μ g brassicasterol, 32.5 μ g campesterol, 8.8 μ g sitosterol and 66.3 μ g stigmasterol) prior to saponification with KOH and extraction with hexane. Comparisons were made between sterol levels in these extracts, sterol levels in extracts made in parallel without addition of the Matreya plant sterol mix, and sterol levels in the derivatised Matreya plant sterol mix.

104% $\pm$ 10% (mean $\pm$ SEM, n = 5) of the total spiked sterols was recovered. However, recovery was not the same for each sterol (Figure 3.5). Brassicasterol recovery was 62% $\pm$ 5% (p < 0.005), suggesting low stability of this sterol during the extraction procedure. Sitosterol was also lower than the total average at 85% $\pm$ 8% (p < 0.01, significant after Bonferroni correction for multiple comparisons). Both campesterol and stigmasterol had higher than 100% recovery. As brassicasterol was absent from BY-2 extracts, recovery of sterols by hexane extraction was subsequently assumed to be 100%.

WT sterol composition

Sterol content of WT BY-2 cells was measured at four days (log phase) and seven days (stationary phase). Total sterol content of 1.83 $\pm$ 0.12 μ g (mg DW)⁻¹ at seven days was not significantly different to 1.49 $\pm$ 0.05 μ g (mg DW)⁻¹ at four days (mean $\pm$ SEM, n $\geq$ 7). However, multivariate analysis of variance (MANOVA) indicated the sterol profiles were significantly different between the two ages (p < 0.0001).

At each time point, the predominant sterols were stigmasterol, sitosterol and campesterol. However, the contributions of individual sterols were different at the two time points (Figure 3.6). In the branch of stigmasterol biosynthesis (Figure 3.7), the proportion of stigmasterol

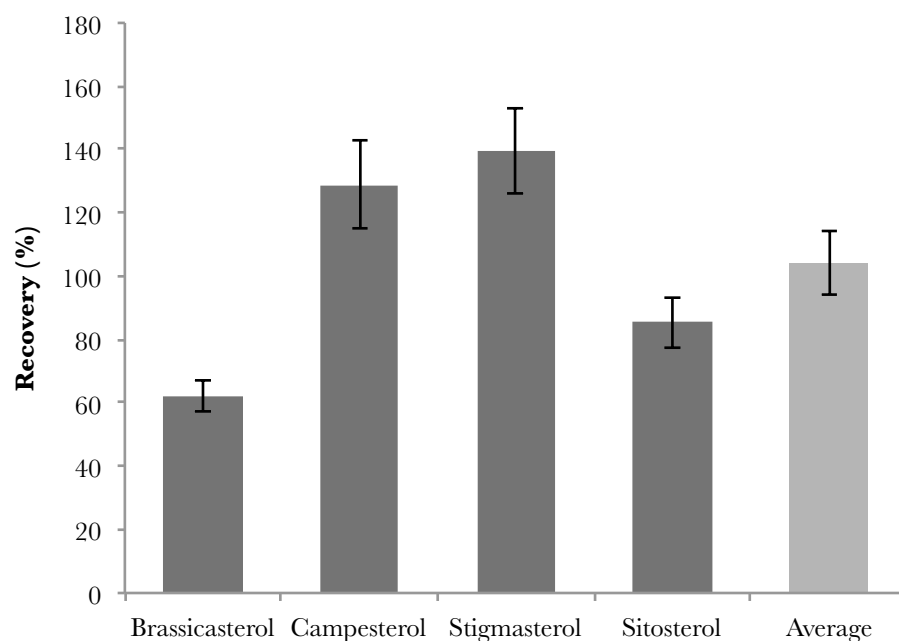
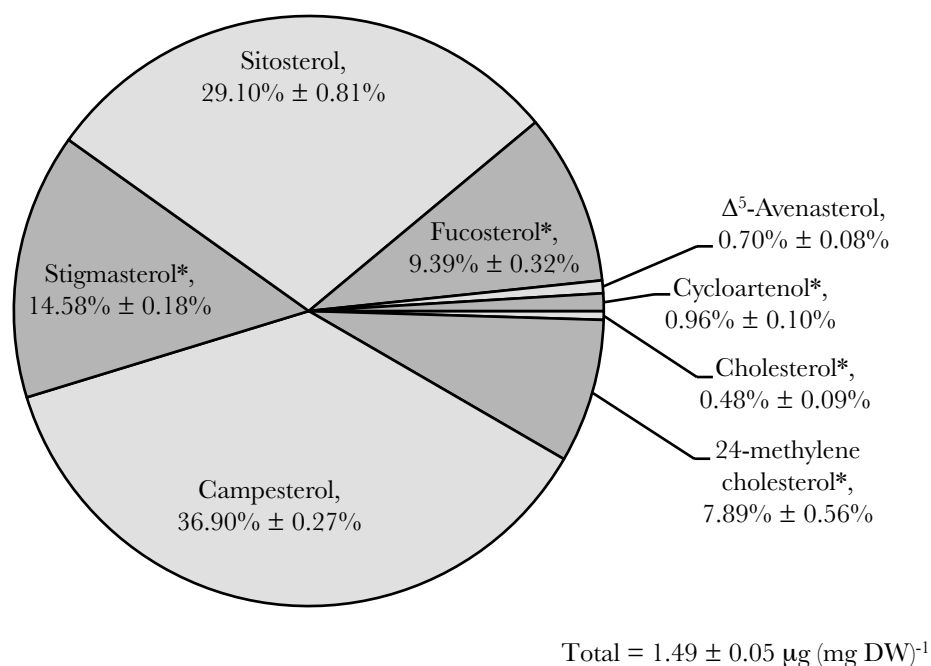


Figure 3.5. Recovery of sterols by alkaline saponification and hexane extraction.

Recovery of sterols was assessed by spiking BY-2 cells (after grinding and freeze-drying) with Matreya plant sterol mix prior to saponification with KOH and extraction with hexane. Values are mean $\pm$ SEM, $n = 5$. The average measurement is the mean total recovery of each replicate.

a)



b)

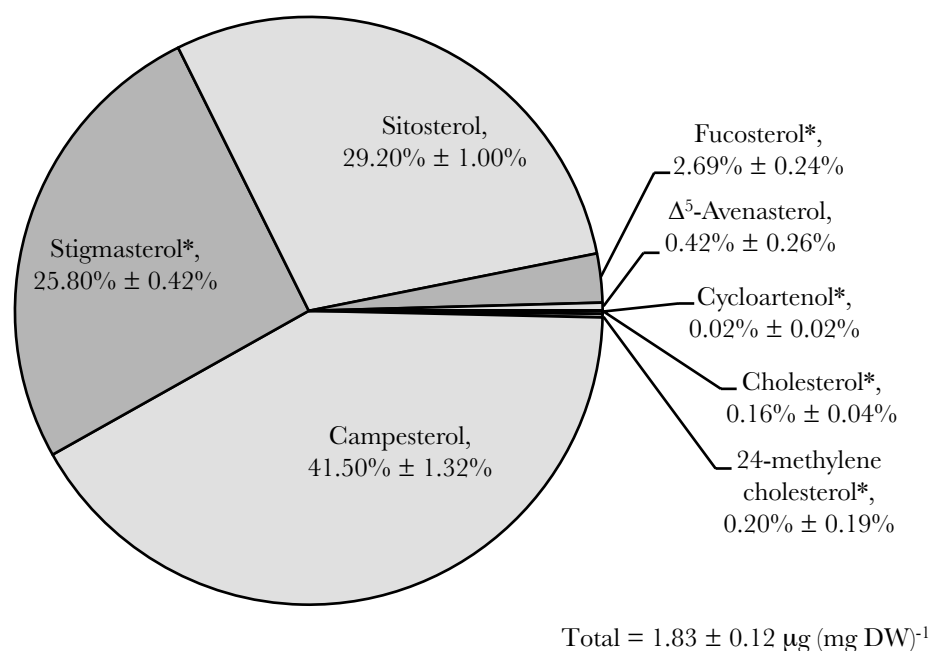


Figure 3.6. Wild-type BY-2 total sterol composition at four and seven days.

Although total sterol content was not significantly different at **(a)** four days compared to **(b)** seven days, sterol composition did change. Values = mean ± SEM, $n \geq 7$; sterols contributing different proportions to the total between the two time points are represented by “*” ($p < 0.005$, significant after Bonferroni correction for multiple comparisons).

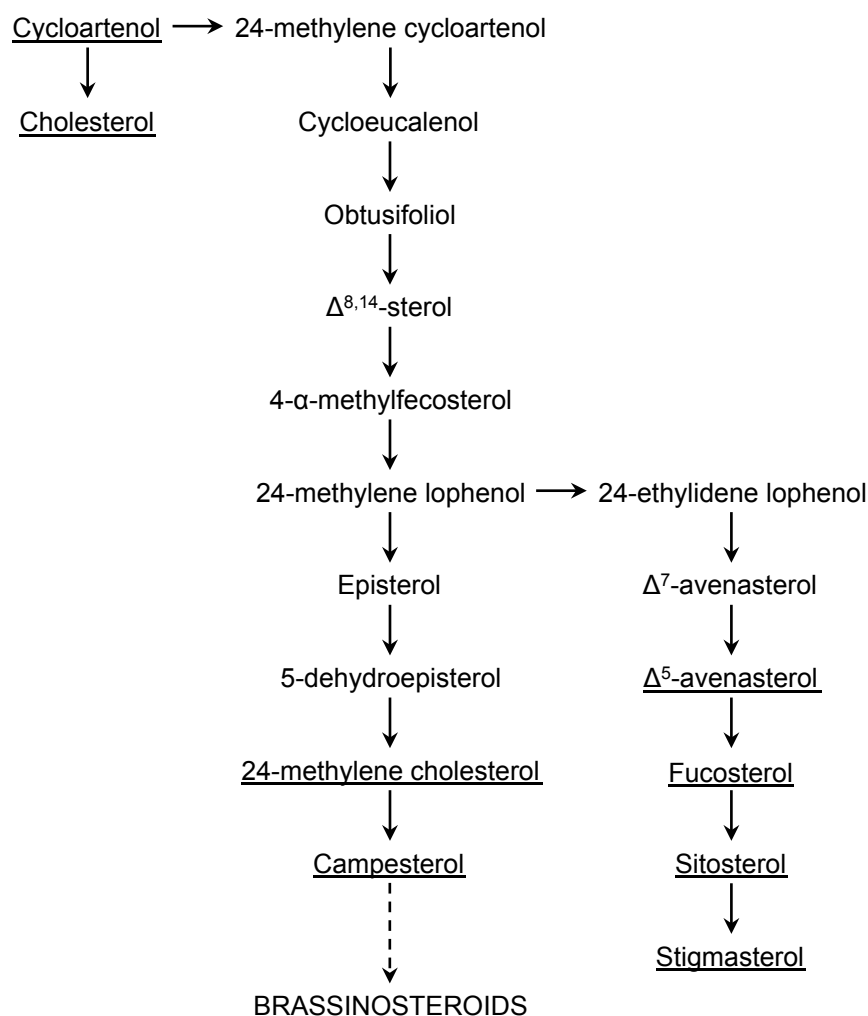


Figure 3.7. Overview of BY-2 sterol biosynthesis.

The figure highlights the three different branches of sterol biosynthesis following the synthesis of cycloartenol. Sterols identified by GC-MS in BY-2 hexane extracts are underlined and represent flux through each of the three branches.

at seven days had increased to 1.8 times the four day proportion, while fucosterol had decreased by 71% ($p < 0.0001$, significant after Bonferroni correction for multiple comparisons). Meanwhile, the contribution of sitosterol was similar, at 29% at each time point. In the campesterol branch, the contribution of 24-methylene cholesterol at seven days was 3% of the contribution at four days ($p < 0.0001$). Further upstream in the sterol biosynthesis pathway, the proportions of cycloartenol and cholesterol were each substantially lower at seven days than at four days (98% ($p < 0.0001$) and 67% ($p < 0.005$) lower, respectively).

The trend in these results is that the proportions of end-of-pathway sterols (stigmasterol and campesterol) are increased at the stationary phase of the growth curve compared to the log phase, and the proportions of intermediate sterols (fucosterol, 24-methylene cholesterol and cycloartenol) are reduced. The exception to this observation is cholesterol, which is an end of pathway sterol but, nevertheless, is a minor sterol representing less than 1% of BY-2 sterol content at each time point.

3.2.2.2. Acetyl-CoA

Acetyl-CoA quantification was desired due to it being a key intermediate between primary metabolism and sterol biosynthesis. Disruption of the interface between primary metabolism and secondary metabolism could result in a change in the steady-state concentration of acetyl-CoA.

Acetyl-CoA in BY-2 extracts was quantified using an enzymatic cycling assay, which followed the reduction of NAD^+ as acetyl-CoA and oxaloacetate are converted into citrate and CoA-SH (Gibon et al., 2002). NAD^+ reduction was identified by increased absorbance at 340 nm and is proportional to acetyl-CoA content.

Assay sensitivity was determined using a dilution series of acetyl-CoA sodium salt. The response was linear between 40 $\mu\text{g } \mu\text{l}^{-1}$ and 400 $\mu\text{g } \mu\text{l}^{-1}$ acetyl-CoA sodium salt (Figure 3.8). This gave the quantification formula:

$$\text{Acetyl-CoA}_{\mu\text{M}} = 168.8 \times (\Delta A_{340} \cdot \text{min}^{-1} + 0.00011)$$

Acetyl-CoA content in WT BY-2 cells after four days of growth was 1.90 ± 0.49 pmol (mg FW)⁻¹ (mean $\pm$ SEM, n = 3).

3.2.2.3. Capsidiol

Chemical elicitation of HMGR activity (pursued in Chapter 6.2.9) in tobacco KY-14 cells leads to a redirection of cytosolic isoprenoid biosynthetic flux away from sterols and into the phytoalexin sesquiterpenoid capsidiol, which is primarily secreted into the culture medium (Chappell and Nable, 1987; Chappell et al., 1989; Chappell et al., 1991).

An underivatised capsidiol standard was analysed by GC-MS and verified by comparison of the mass spectrum with reference data supplied with the capsidiol standard and with published mass spectra (Fuchs et al., 1983; Wibberley et al., 1994; Takahashi et al., 2005; Mialoundama et al., 2009). Comparison of the integrated capsidiol GC-MS peak with the integrated peak of the internal standard, pentadecane, revealed a linear response at injected capsidiol concentrations greater than 0.2 $\mu\text{g } \mu\text{l}^{-1}$ ($R^2 = 0.976$; Figure 3.9a). A similar response was also apparent when the integrated peak of m/z 221 of capsidiol was compared against the integrated peak of m/z 212 of pentadecane ($R^2 = 0.976$; Figure 3.9b).

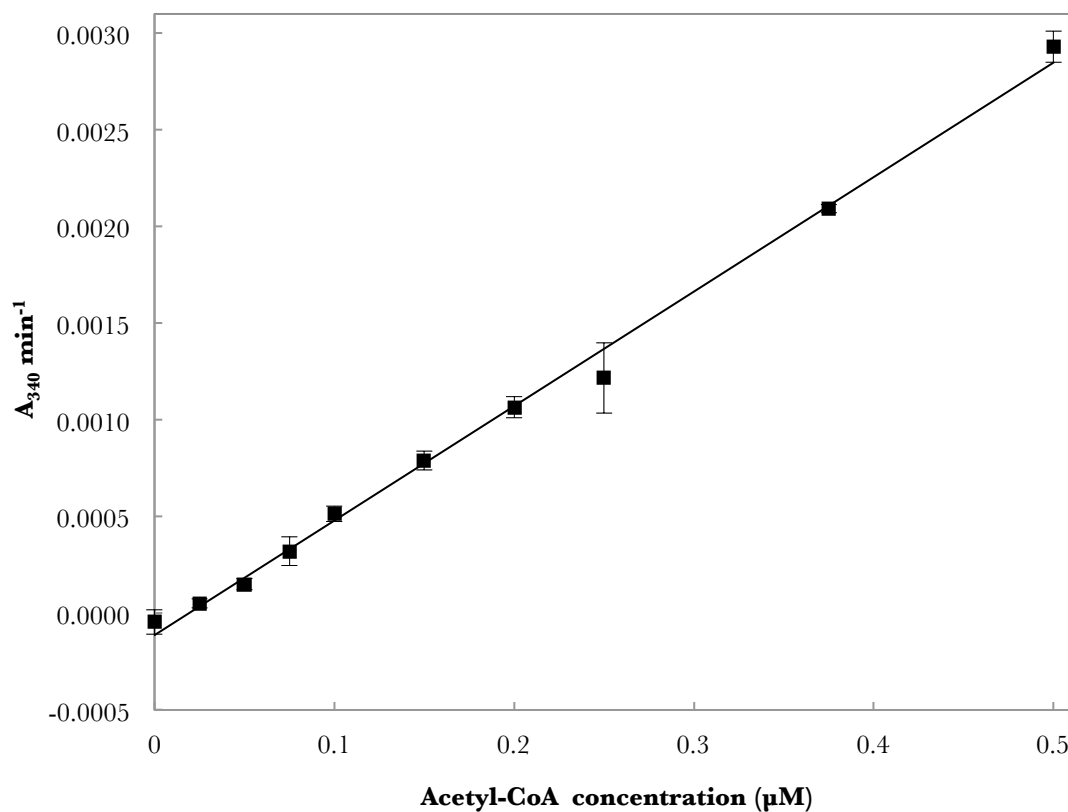


Figure 3.8. Calibration curve for acetyl-CoA quantified by an enzymatic cycling assay.

Increase in absorbance at 340 nm, a measure of NAD⁺ reduction, was linear at acetyl-CoA concentrations greater than 0.05 μM. R^2 for all replicates = 0.982. Data points are mean $\pm$ SEM, $n = 3$.

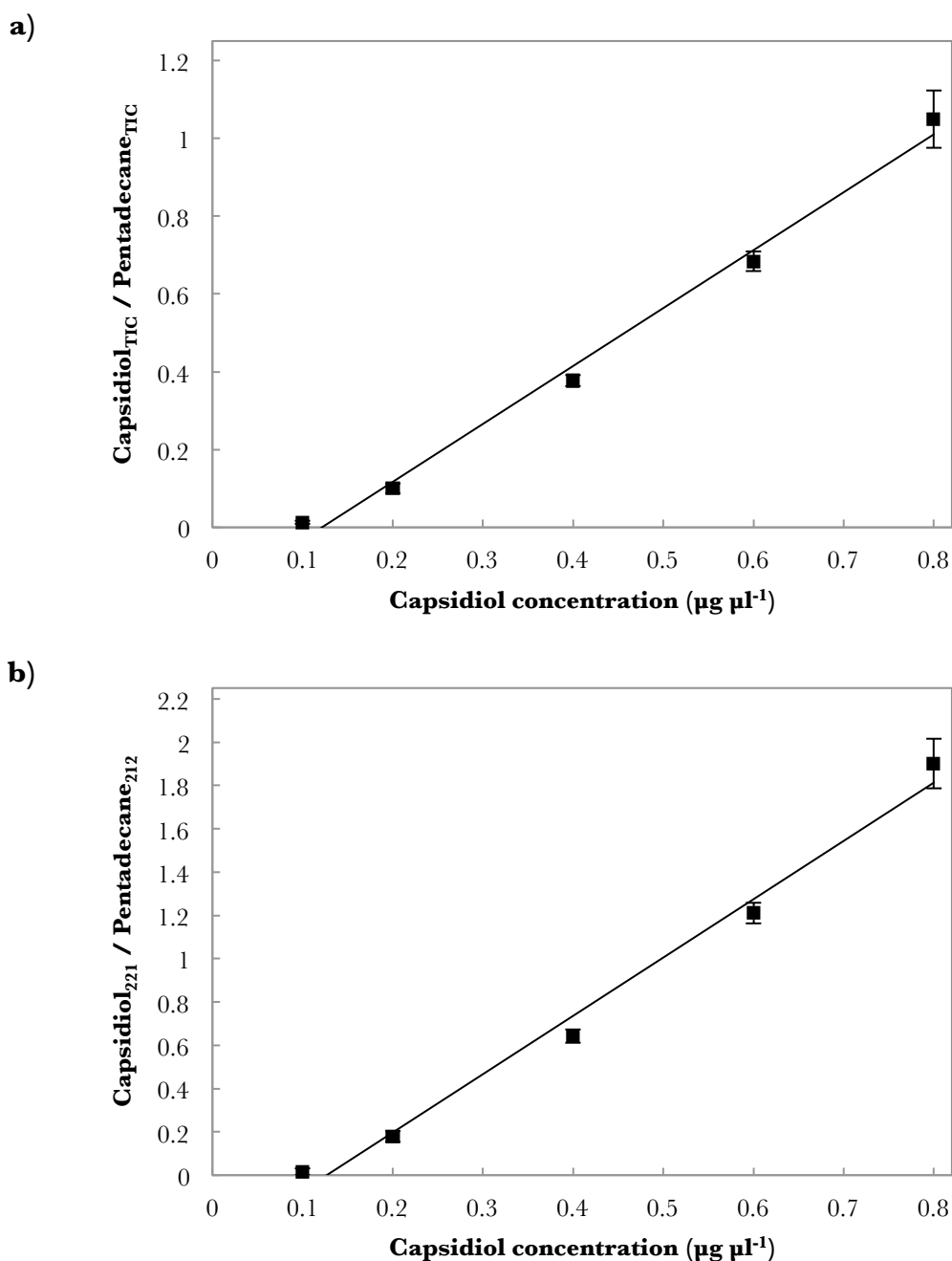


Figure 3.9. Calibration of GC-MS response to capsidiol concentration.

A range of capsidiol concentrations was analysed by GC-MS using $0.2 \mu\text{g } \mu\text{l}^{-1}$ pentadecane as an internal standard. **(a)** Peak area when all ions were quantified (TIC). **(b)** Peak area following filtering in ChemStation for pentadecane m/z 212 and capsidiol m/z 221. Each graph shows linearity at concentrations greater than $0.2 \mu\text{g } \mu\text{l}^{-1}$. In each graph, R^2 for all replicates = 0.976. a) $y = 1.488x - 0.178$, b) $y = 2.691x - 0.334$. Data points represent mean $\pm$ SEM, $n = 3$.

Identification of capsidiol is complicated by its mass spectrum containing many m/z values at similar intensities (Figure 3.10). This, coupled with relatively noisy measurements of BY-2 CHCl₃ extracts, led to the use of the quantification formula based on capsidiol m/z 221 and pentadecane m/z 212, values which were rare in the GC-MS data from BY-2 CHCl₃ extracts:

$$Capsidiol_{\mu g \mu l^{-1}} = 0.372 \times \left(\frac{Capsidiol_{Area (221)}}{Pentadecane_{Area (212)}} + 0.334 \right)$$

Initial experiments with BY-2 and culture filtrate CHCl₃ extracts revealed no capsidiol.

The capsidiol extraction method was validated by adding a known quantity of capsidiol standard to BY-2 cells or culture filtrate prior to CHCl₃ extraction. Capsidiol content of these spiked extracts was compared with unspiked extracts. 79% ± 4% (mean ± SEM, n = 3) capsidiol was recovered from BY-2 cells, while 82% ± 4% was recovered from culture filtrate.

3.2.2.4. GC-MS-based metabolomics

Altering enzyme activity in one branch of metabolism can affect metabolite levels in distal regions of metabolism. Examples of this phenomenon include the accumulation of anthocyanin in *Arabidopsis* (*Arabidopsis thaliana*) with decreased ATP-citrate lyase activity (Fatland et al., 2005), reduction of carotenoid and tocopherol levels in tobacco with decreased transketolase activity (Henkes et al., 2001), and changes in amino acid levels in California poppy (*Eschscholzia californica*) root cultures following genetic manipulation of the benzophenanthridine alkaloid biosynthesis pathway (Park et al., 2002; Park et al., 2003).

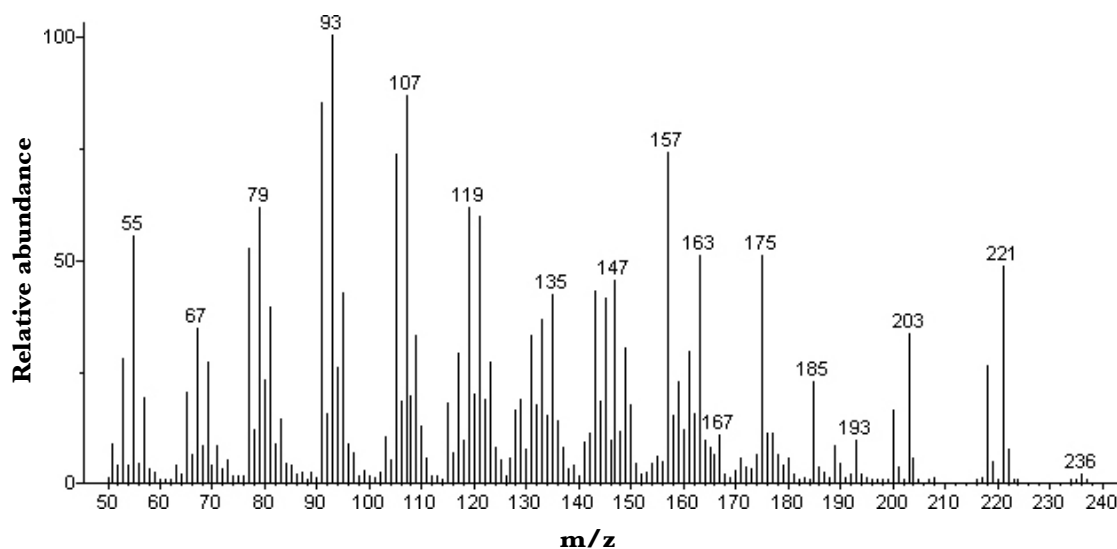


Figure 3.10. GC-MS ion chromatogram of an underivatised capsidiol standard (34.60 to 34.74 min).

Underivatised capsidiol standard was analysed by GC-MS. The compound eluting at 34.60 to 34.74 min was verified to be capsidiol by comparisons with published mass isotopomer distributions (Fuchs et al., 1983; Wibberley et al., 1994; Takahashi et al., 2005; Mialoundama et al., 2009) and with the mass isotopomer distribution on the datasheet that accompanied the standard.

Use of TagFinder for high-throughput identification of GC-MS spectra

To determine some of the metabolomic effects of genetic manipulations applied in Chapter 4, a broad-spectrum GC-MS-based metabolomics approach was employed using TagFinder software (Luedemann et al., 2008). TagFinder works by aligning chromatograms from several samples on the basis of the elution times of alkanes of different masses that are added to the samples during the derivatisation procedure. After alignment, the software puts analytes into bins based on their retention time. Analytes are then identified simultaneously in all experimental samples on the basis of retention time and mass spectrum.

The output from TagFinder is a table of rows of mass tags arranged by time-group and analyte identity, and columns of intensity values for each sample. The most abundant mass tag for each analyte was used for calculation of relative abundances. The use of an internal standard (ribitol) added prior to metabolite extraction allowed abundance to be normalised to DW. Abundance values of each analyte in negative controls of the methanol extraction procedure (without cell material), which were analysed simultaneously, were then subtracted from each test sample. This allowed metabolite profiles to be compared between conditions (in this case cell culture age).

Different analytes with identical mass spectra are not easily distinguished using TagFinder. These were instead verified by manual study of the GC-MS chromatograms. For instance, the aldopentoses xylose, lyxose, arabinose and ribose are indistinguishable from each other on the basis of their mass spectra. 1MEOX 4TMS derivatives of these compounds elute with tag-times of 1636.65, 1650.19, 1651.53 and 1669.25, respectively. They were assigned to time groups 236, 237, 238 and 240 after manually verifying the presence of the four elution peaks with matching spectra. Further manual curation was required when time groups failed to align across all samples. For instance, sucrose (8TMS) was assigned to time groups 536 and 544. In all cases except replicate two of the six-day-old culture set, the sucrose tag (m/z 361)

was most abundant in time group 544, but this m/z value was highest in time group 536 of replicate two of the six-day-old culture set.

Univariate statistical analysis of metabolomics data

Applying the metabolomics approach to WT BY-2 methanol extracts from logarithmic phase to early stationary phase (four, five and six days) led to the identification of 85 metabolites comprising 104 different analytes. Pyruvic acid (1MEOX) (1TMS), α -1-*O*-methyl-glucopyranoside (4TMS), heptadecanoic acid (1TMS), isomaltose (1MEOX) (8TMS) BP and cycloartenol (1TMS) were identified in fewer than two samples and were omitted from further analyses. Ribitol (5TMS) was not identified in the second replicate of day five measurements. As a result, this sample was omitted from further analyses. Where an analyte was not identified in an extract, the abundance of that analyte was assumed to be 0. The intensity of the ribitol (5TMS) mass-tag 217 did not vary between ages (Appendix 3.3). This confirmed the suitability of this tag for normalisation of other analyte tag intensities.

99 analytes comprising 79 metabolites were identified in at least two samples (Table 3.4). β -alanine (3TMS) (Figure 3.11a) and ethanolamine (3TMS) (Figure 3.11b) were each significantly more abundant at six days than at either four days or five days ($p < 0.0005$, significant after Bonferroni-correction for multiple comparisons). Levels of threonic acid (4TMS), by contrast, at six days were $40\% \pm 5\%$ of the concentration at four days ($p < 0.0005$) (Figure 3.11c). There were no significant differences in the abundance of any metabolite between four days and five days.

Normalised concentrations of individual methanol-soluble metabolites as proportions of DW at four days were on average 0.59 ± 0.05 and 0.54 ± 0.04 times the levels at five days and six days, respectively ($p < 0.0001$), with only 17 out of 99 analytes having mean concentrations

Table 3.4. Metabolomics data for wild-type time-course experiment.

Values for each analyte were normalised to the dataset mean for that analyte, after intensities were first normalised to ribitol and DW. Values that are significantly different to six-day values are **bold** (significant after Bonferroni correction for multiple comparisons with an initial critical p-value of 0.05). There were no significant differences between four and five days. Values are expressed as mean $\pm$ standard error, n = 4 for days four and six, n = 3 for day five.

Analyte	Days post-subculture		
	4	5	6
Aconitic acid, cis- (3TMS)	0.42 $\pm$ 0.33	1.64 $\pm$ 1.59	0.94 $\pm$ 0.88
Alanine (2TMS)	0.89 $\pm$ 0.89	0.92 $\pm$ 0.92	1.19 $\pm$ 1.19
Alanine (3TMS)	0.72 $\pm$ 0.28	0.66 $\pm$ 0.17	1.62 $\pm$ 0.52
Alanine [+CO ₂] (2TMS)	0.48 $\pm$ 0.28	0.60 $\pm$ 0.11	1.93 $\pm$ 0.56
Alanine, beta- (3TMS)	0.63 $\pm$ 0.06	0.69 $\pm$ 0.06	1.68 $\pm$ 0.07
Arabinose (1MEOX) (4TMS) MP	1.03 $\pm$ 0.10	1.08 $\pm$ 0.20	0.89 $\pm$ 0.10
Asparagine (2TMS)	0.74 $\pm$ 0.10	0.92 $\pm$ 0.37	1.35 $\pm$ 0.53
Asparagine (3TMS)	0.12 $\pm$ 0.07	1.34 $\pm$ 0.81	1.54 $\pm$ 0.58
Asparagine [-H ₂ O] (2TMS)	0.67 $\pm$ 0.30	1.02 $\pm$ 0.18	1.30 $\pm$ 0.31
Aspartic acid (2TMS)	0.84 $\pm$ 0.49	0.88 $\pm$ 0.25	1.28 $\pm$ 0.47
Aspartic acid (3TMS)	0.75 $\pm$ 0.61	1.67 $\pm$ 1.44	0.57 $\pm$ 0.45
Butanoic acid, 2-amino- (2TMS)	0.75 $\pm$ 0.75	0.80 $\pm$ 0.80	1.45 $\pm$ 1.45
Butanoic acid, 2,4-dihydroxy- (3TMS)	1.02 $\pm$ 0.10	1.19 $\pm$ 0.25	0.78 $\pm$ 0.13
Butanoic acid, 4-amino- (3TMS)	0.82 $\pm$ 0.18	1.02 $\pm$ 0.44	1.16 $\pm$ 0.21
Butanoic acid, 4-hydroxy- (2TMS)	0.37 $\pm$ 0.38	1.52 $\pm$ 1.18	1.11 $\pm$ 0.36
Butyro-1,4-lactam (1TMS)	0.45 $\pm$ 0.28	1.45 $\pm$ 0.57	1.10 $\pm$ 0.48
Campesterol (1TMS)	0.45 $\pm$ 0.39	1.15 $\pm$ 0.65	1.39 $\pm$ 1.03
Citric acid (4TMS)	0.57 $\pm$ 0.23	1.05 $\pm$ 0.31	1.38 $\pm$ 0.18
Dehydroascorbic acid dimer (2MEOX) MP	0.75 $\pm$ 0.09	1.00 $\pm$ 0.29	1.25 $\pm$ 0.20
Eicosanoic acid (1TMS)	0.39 $\pm$ 0.32	1.71 $\pm$ 0.71	0.90 $\pm$ 0.69
Erythronic acid (4TMS)	0.96 $\pm$ 0.06	1.09 $\pm$ 0.16	0.95 $\pm$ 0.08
Ethanolamine (3TMS)	0.20 $\pm$ 0.11	0.37 $\pm$ 0.11	2.44 $\pm$ 0.11
Fructose (1MEOX) (5TMS) BP	0.85 $\pm$ 0.36	0.84 $\pm$ 0.32	1.30 $\pm$ 0.52
Fructose (1MEOX) (5TMS) MP	0.84 $\pm$ 0.36	0.83 $\pm$ 0.39	1.32 $\pm$ 0.55
Fucosterol (1TMS)	0.44 $\pm$ 0.44	1.84 $\pm$ 1.04	0.72 $\pm$ 0.72
Fumaric acid (2TMS)	0.68 $\pm$ 0.15	1.17 $\pm$ 0.42	1.15 $\pm$ 0.37
Galactinol (9TMS)	0.70 $\pm$ 0.31	1.02 $\pm$ 0.24	1.28 $\pm$ 0.47
Galactopyranoside, 1-O-methyl-, alpha- (4TMS)	0.28 $\pm$ 0.18	0.71 $\pm$ 0.38	2.01 $\pm$ 0.73
Galactose-6-phosphate (1MEOX) (6TMS) early peak (38.3 min)	1.01 $\pm$ 0.37	1.55 $\pm$ 0.17	0.45 $\pm$ 0.16
Galactose-6-phosphate (1MEOX) (6TMS) late peak (38.9 min)	1.01 $\pm$ 0.35	1.63 $\pm$ 0.10	0.36 $\pm$ 0.13
Gluconic acid (6TMS)	0.85 $\pm$ 0.36	1.41 $\pm$ 0.41	0.74 $\pm$ 0.16
Glucopyranose, D- (5TMS)	0.28 $\pm$ 0.20	0.91 $\pm$ 0.51	1.81 $\pm$ 0.44
Glucose (1MEOX) (5TMS) BP	1.09 $\pm$ 0.43	1.09 $\pm$ 0.34	0.82 $\pm$ 0.32
Glucose (1MEOX) (5TMS) MP	0.54 $\pm$ 0.33	1.53 $\pm$ 0.69	0.93 $\pm$ 0.68
Glucose-6-phosphate (1MEOX) (6TMS) MP	1.09 $\pm$ 0.24	1.54 $\pm$ 0.07	0.37 $\pm$ 0.12
Glucose-6-phosphate (6TMS)	0.21 $\pm$ 0.21	0.81 $\pm$ 0.40	1.98 $\pm$ 0.40
Glutamic acid (2TMS)	1.00 $\pm$ 0.33	0.62 $\pm$ 0.41	1.39 $\pm$ 0.71
Glutamic acid (3TMS)	0.72 $\pm$ 0.20	1.14 $\pm$ 0.31	1.14 $\pm$ 0.02

Analyte	Days post-subculture		
	4	5	6
Glutamine [-H ₂ O] (2TMS) BP	0.41 ± 0.17	1.29 ± 0.82	1.29 ± 0.54
Glutamine [-H ₂ O] (3TMS) MP	0.56 ± 0.24	0.46 ± 0.09	1.97 ± 1.17
Glutamine, DL- (3TMS)	0.15 ± 0.15	1.25 ± 0.97	1.60 ± 0.70
Glutamine, DL- (4TMS)	0.60 ± 0.57	1.28 ± 0.72	1.12 ± 0.62
Glutaric acid, 2-oxo- (1MEOX) (2TMS) MP	0.83 ± 0.16	0.64 ± 0.11	1.53 ± 0.18
Glyceric acid (3TMS)	1.01 ± 0.10	1.22 ± 0.29	0.77 ± 0.11
Glycerol-2-phosphate (4TMS)	-0.83 ± 1.17	2.95 ± 2.42	0.88 ± 2.16
Glycerol-3-phosphate (4TMS)	0.93 ± 0.05	1.27 ± 0.12	0.80 ± 0.17
Glycerophosphoglycerol (5TMS)	1.31 ± 0.55	0.88 ± 0.50	0.81 ± 0.58
Glycine (2TMS)	0.13 ± 0.13	1.61 ± 1.61	1.26 ± 1.26
Glycine (3TMS)	0.55 ± 0.15	0.82 ± 0.25	1.63 ± 0.24
Hexadecanoic acid (1TMS)	0.62 ± 0.10	1.34 ± 0.20	1.04 ± 0.29
Homoserine (3TMS)	0.55 ± 0.16	0.96 ± 0.24	1.49 ± 0.24
Inositol, myo- (6TMS)	0.87 ± 0.11	1.15 ± 0.35	0.98 ± 0.15
Isoleucine (1TMS)	0.66 ± 0.26	1.21 ± 0.28	1.13 ± 0.38
Lactic acid, DL- (2TMS)	0.03 ± 0.03	0.01 ± 0.01	2.96 ± 2.96
Leucine (1TMS)	0.61 ± 0.25	1.07 ± 0.24	1.32 ± 0.44
Lyxonic acid (5TMS)	1.21 ± 0.28	0.87 ± 0.19	0.92 ± 0.33
Lyxose (1MEOX) (4TMS) MP	0.72 ± 0.28	1.24 ± 0.22	1.04 ± 0.39
Malic acid (3TMS)	0.85 ± 0.18	0.89 ± 0.21	1.26 ± 0.24
Malic acid, 2-methyl- (3TMS)	1.15 ± 0.09	1.21 ± 0.22	0.64 ± 0.08
Maltose (1MEOX) (8TMS) BP	0.67 ± 0.23	1.12 ± 0.03	1.21 ± 0.41
Maltose (1MEOX) (8TMS) MP	0.92 ± 0.37	1.62 ± 0.51	0.46 ± 0.17
Methionine (1TMS)	0.21 ± 0.10	0.51 ± 0.26	2.28 ± 0.86
Methionine (2TMS)	0.21 ± 0.10	0.47 ± 0.23	2.32 ± 0.36
myo-Inositol-1-phosphate (7TMS)	0.78 ± 0.26	1.46 ± 0.11	0.76 ± 0.30
Norleucine (2TMS)	1.06 ± 0.59	0.85 ± 0.25	1.09 ± 0.54
Octadecadienoic acid, n- (1TMS)	0.46 ± 0.25	1.28 ± 0.23	1.26 ± 0.63
Octadecan-1-ol, n- (1TMS)	0.60 ± 0.15	1.36 ± 0.88	1.05 ± 0.60
Octadecanoic acid (1TMS)	0.64 ± 0.29	1.34 ± 0.49	1.03 ± 0.53
Ornithine-1,5-lactam (2TMS)	2.22 ± 0.77	0.78 ± 0.78	0.00 ± 0.00
Phenylalanine (2TMS)	0.50 ± 0.15	1.15 ± 0.27	1.35 ± 0.14
Phosphoric acid (3TMS)	1.52 ± 0.37	1.05 ± 0.23	0.43 ± 0.15
Putrescine (4TMS)	0.62 ± 0.18	0.96 ± 0.30	1.42 ± 0.49
Pyroglutamic acid (2TMS)	0.76 ± 0.18	0.91 ± 0.28	1.33 ± 0.27
Ribonic acid (5TMS) early peak (29.1 min)	0.96 ± 0.13	1.01 ± 0.23	1.03 ± 0.10
Ribonic acid (5TMS) late peak (29.2 min)	0.66 ± 0.20	1.13 ± 0.22	1.21 ± 0.11
Ribose (1MEOX) (4TMS) BP	0.88 ± 0.10	1.25 ± 0.32	0.87 ± 0.13
Salicin (5TMS)	-1.48 ± 1.73	1.89 ± 3.14	2.58 ± 4.10
Serine (2TMS)	0.52 ± 0.21	0.98 ± 0.28	1.50 ± 0.47
Serine (3TMS)	0.60 ± 0.32	0.59 ± 0.25	1.81 ± 0.92
Serine (4TMS)	0.81 ± 0.45	0.78 ± 0.29	1.42 ± 0.47
Serine, O-acetyl- (2TMS)	0.42 ± 0.15	0.79 ± 0.23	1.79 ± 0.17
Sitosterol, beta- (1TMS)	0.45 ± 0.37	1.13 ± 0.61	1.42 ± 1.08
Stigmasterol (1TMS)	0.42 ± 0.37	1.00 ± 0.66	1.58 ± 1.18
Succinic acid (2TMS)	0.64 ± 0.08	0.71 ± 0.09	1.65 ± 0.24

Analyte	Days post-subculture		
	4	5	6
Sucrose (8TMS)	0.53 ± 0.29	1.48 ± 0.65	0.99 ± 0.59
Tagatose (1MEOX) (5TMS) BP	0.42 ± 0.27	0.07 ± 0.07	2.50 ± 2.50
Threonic acid (4TMS)	1.16 ± 0.06	1.37 ± 0.18	0.47 ± 0.06
Threonine (3TMS)	0.68 ± 0.29	0.91 ± 0.33	1.40 ± 0.48
Threonine, DL- (2TMS)	0.62 ± 0.22	1.06 ± 0.20	1.32 ± 0.42
Turanose (1MEOX) (8TMS) BP	0.89 ± 0.32	1.37 ± 0.17	0.74 ± 0.25
Tyrosine (3TMS)	0.20 ± 0.12	1.18 ± 0.53	1.62 ± 0.55
Uracil (2TMS)	0.88 ± 0.18	1.30 ± 0.26	0.83 ± 0.27
Uric acid (4TMS)	0.25 ± 0.15	1.21 ± 0.34	1.54 ± 0.68
Uridine (4TMS)	1.14 ± 0.66	1.86 ± 1.47	0.00 ± 0.00
Valine (1TMS)	0.01 ± 0.01	2.96 ± 2.96	0.03 ± 0.03
Valine (2TMS)	0.64 ± 0.29	0.91 ± 0.39	1.44 ± 0.46
Xylitol (5TMS)	0.74 ± 0.19	1.32 ± 0.22	0.94 ± 0.22
Xylose (1MEOX) (4TMS) MP	1.10 ± 0.63	0.50 ± 0.05	1.40 ± 0.68
Xylulose-5-phosphate (1MEOX) (5TMS) BP	1.38 ± 0.31	1.31 ± 0.35	0.31 ± 0.05

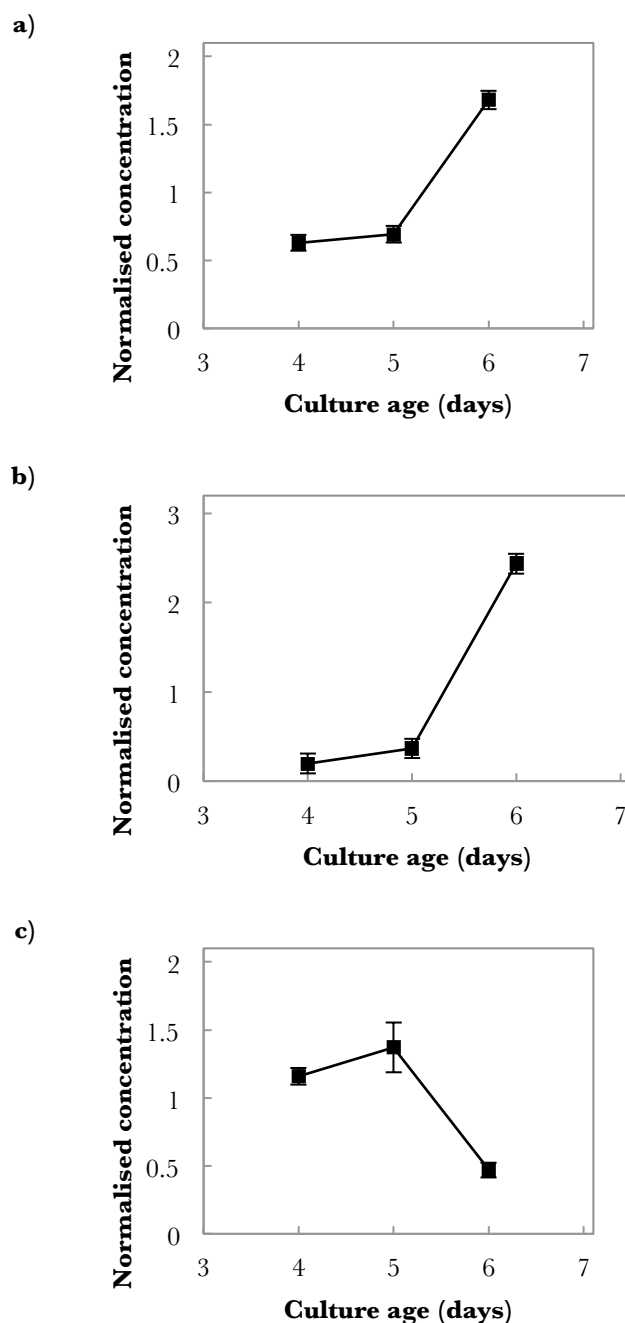


Figure 3.11. Age-related metabolite changes in wild-type BY-2 cell cultures.

(a) β -alanine (3TMS) and **(b)** ethanolamine (3TMS) in methanol extracts from six-day-old cultures were more abundant than extracts from four- and five-day-old cultures. **(c)** Threonic acid (4TMS) was less abundant in extracts from six-day-old cultures than from four-day-old cultures. ($p < 0.0005$, significant after Bonferroni correction for multiple comparisons. Concentrations were normalised to the internal standard (ribitol), biomass DW and the mean values of the dataset. Data points = mean $\pm$ SEM, $n = 4$ for days four and six, $n = 3$ for day five.)

than the average of the data set (compared with 61 being higher at five days and 66 at six days).

Multivariate statistical analysis of metabolomics data

The metabolome data was further analysed by principal component analysis (PCA). Unit variance scaling was used to give equal weighting to analytes regardless of the relative mean levels compared to other analytes or the degree of variance from that mean.

PCA identified three principal components, which explained 31.2%, 27.1% and 18.2% of the variance between samples (Figure 3.12). The remaining 23.5% of variance was not attributable to any statistically significant components. MANOVA of the principal scores for each age group indicated significant differences between the age groups ($p < 0.0001$). Further MANOVAs indicated significant differences between the metabolomes at days four and six ($p < 0.05$), and days five and six ($p < 0.05$), while days four and five were not significantly different. Analysis of variance did not indicate significant differences between the age groups within any individual component.

Further PCA was performed using pairwise comparisons of age-groups. This identified individual principal components for which six-day-old cultures had significantly different scores to four- and five-day-old cultures (Appendix 3.4). Loadings provide information on the contribution of each analyte to the specific principal component. Loading data therefore suggested that certain analytes were enriched at six days compared to four and five days. 49 of the 99 analytes were relatively enriched at six days compared to four days. These 49 analytes comprised approximately half of each metabolite class (Table 3.5). Meanwhile, comparisons of five and six day data suggested that 36 analytes were enriched at six days, with 21 of these analytes being amino acids. This represents 60% of the amino acid analytes. With the

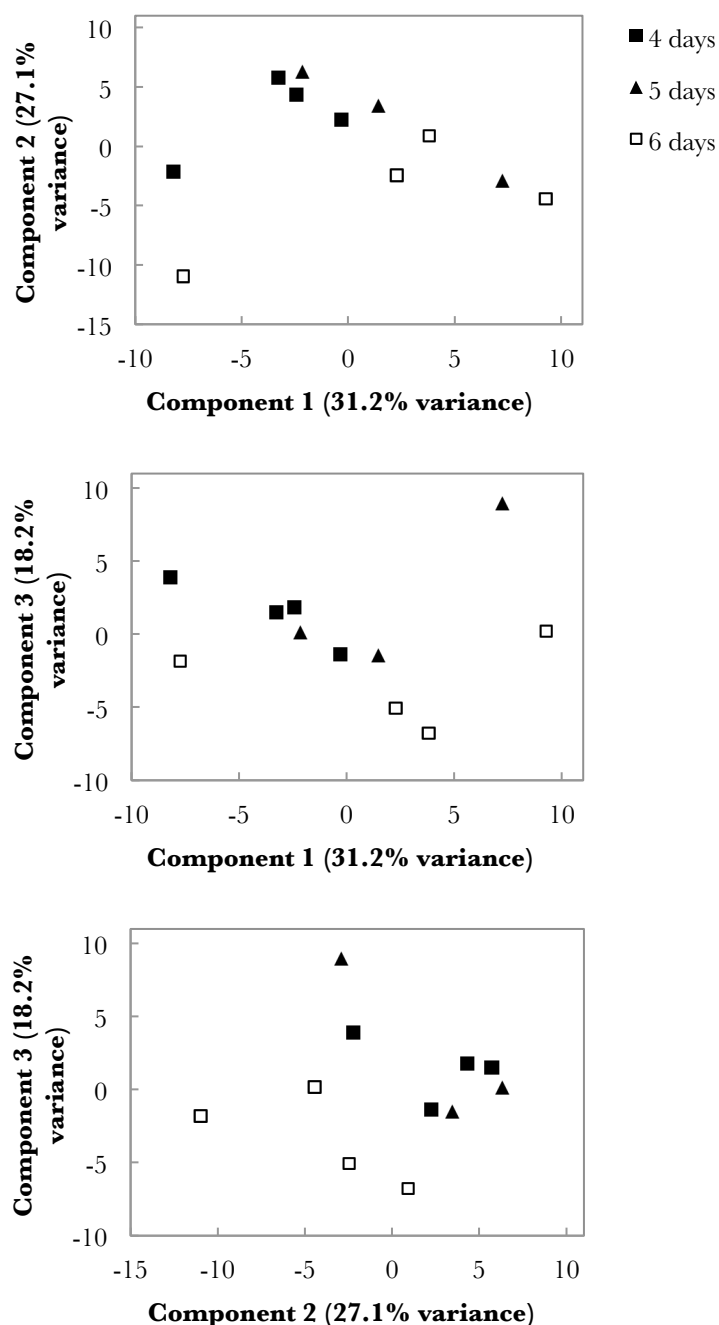


Figure 3.12. Principal component analysis of wild-type BY-2 metabolome data.

Score plots of the three statistically significant principal components of metabolome data are presented. Multivariate analysis of variance (MANOVA) of this data indicates significant differences between metabolomes at four and six days ($p < 0.05$), and five and six days ($p < 0.05$). Cultures harvested after four and five days were not significantly different. There were no significant differences between any age groups confined to a single principal component, as determined by analysis of variance (ANOVA). Further pairwise PCA is presented in Appendix 3.4.

Table 3.5. Categorisation of analytes suggested by principal component analysis to alter in relative abundance as culture growth slows.

99 analytes were used in pairwise PCA of culture age. Analytes were subcategorised based on their metabolite type for easier interpretation. Analytes with loadings less than -0.05 for principal component two in four versus six-day comparisons were considered to be enriched at six days relative to four days, while those with loadings greater than 0.05 were considered to be depleted. Analytes with loadings greater than 0.05 for principal component three of five versus six-day comparisons were considered to be enriched at six days compared to five days, while those with loadings less than -0.05 were considered to be depleted. Statistical analyses of PCA scores from comparisons of four- and five-day-old cultures did not reveal significant differences between the metabolomes at those two ages. A full list of loading scores for all analytes for the diagnostic components is provided in Appendix 3.4.

Identity	All	Enriched at six days compared to:		Depleted at six days compared to:	
		Four days	Five days	Four days	Five days
Amino acids	35	17	21	5	5
Organic acids	24	14	4	3	12
Sterols	4	2	3	1	0
Sugars	19	10	2	5	9
Triacylglycerol components	9	3	2	0	5
Other metabolites	8	3	4	4	3
Total	99	49	36	17	33

exception of methionine, phenylalanine, tyrosine and succinate, there were no metabolites that were consistently implied by loading scores to be increased in abundance at six days compared to four-day and five-day measurements. Analytes suggested by loading scores to be depleted at six days compared to four and five days were also not confined to particular metabolite classes. However, there was greater agreement between the PCAs comparing six days with four days and six days with five days. Loadings from each suggested depletion at six days of 2,4-dihydroxy-butanoate, galactose-6-phosphate, gluconate, glycerate, glycerol-3-phosphate, 2-methyl-malate, myo-inositol-1-phosphate, phosphoric acid, threonate, uridine and xylulose-5-phosphate.

3.2.3. Optimisation of enzyme assays and measurements of wild-type BY-2 cell enzyme activities

Increased activities of citrate synthase (CS) and HMGR are pursued in Chapters 4 and 6, respectively. Prior to targeting these enzymes, experiments were performed to determine suitable means of assaying the activity of each enzyme.

3.2.3.1. Citrate synthase

CS was assessed using an assay that coupled acetyl-CoA and oxaloacetate consumption by CS to production of oxaloacetate by the action of exogenously supplied malate dehydrogenase on malate and 3-acetylpyridine adenine dinucleotide (APAD) (Stitt et al., 1989). Reduction of APAD was detected as an increase in absorbance at 365 nm.

A dilution series of desalted BY-2 protein extracts was analysed to determine a linear range of ΔA_{365} response. The response was linear for at least 3 min with ΔA_{365} up to 0.037 min^{-1} , corresponding to 4.1 mM min^{-1} acetyl-CoA consumption (Figure 3.13). Triplicate assays of extraction triplicates from the same culture demonstrated that the assay had a coefficient of variation of 0.096 ± 0.23 (mean $\pm$ SD) and that protein extraction had a coefficient of variation, with respect to CS activity, of 0.071. WT CS activity at four days was measured at $13.5 \pm 0.9 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ (mean $\pm$ SEM, $n = 6$).

3.2.3.2. HMGR

Different HMGR assay protocols were attempted in order to find a reliable method for measuring HMGR activity in BY-2 extracts. Initially, the reduction in A_{340} was used as a proxy for NADPH oxidation as HMG-CoA is reduced (Asadollahi et al., 2010). A dilution series of desalted total protein extract from four-day-old BY-2 cultures did not produce a linear response, as addition of HMG-CoA to the assay did not significantly alter the detectable rate of NADPH oxidation (Appendix 3.5). This assay was similarly insensitive to HMGR activity in microsomal extracts: following addition of HMG-CoA to $63 \text{ }\mu\text{g}$ of microsomal protein, A_{340} dropped by only 0.001 AU, the smallest measurable unit change. In order to allow a more concentrated sample to be assayed, reaction volumes were scaled down by 90% and ΔA_{340} was recorded using a microplate reader. Addition of HMG-CoA to a dilution series of microsomal extract again did not significantly alter the detectable rate of NADPH oxidation (Appendix 3.5).

Following these results, a stopped assay measuring the conversion of *S*-[3- ^{14}C]-HMG-CoA to *S*-[3- ^{14}C]-mevalonate was tested. This involved separation by thin layer chromatography (TLC) followed by measurement of the relative radioactivity at the origin (unreacted

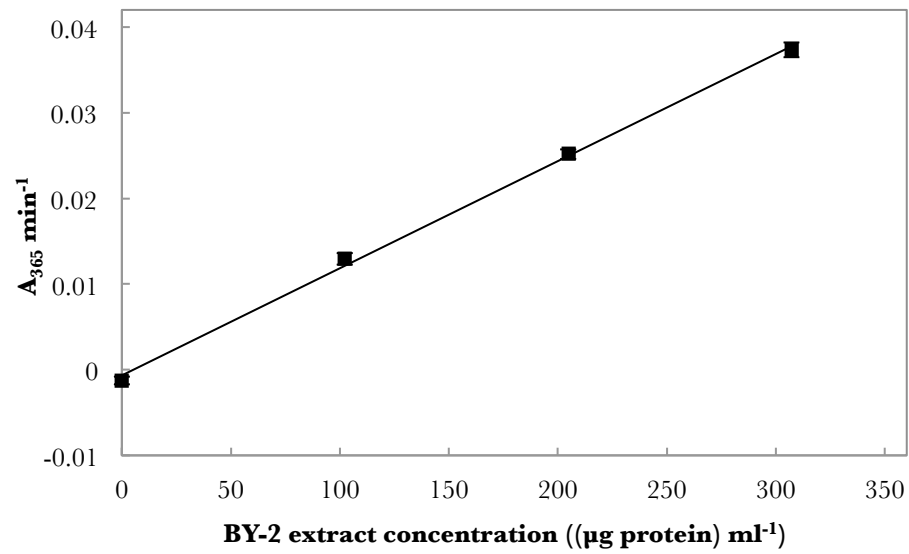


Figure 3.13. Citrate synthase assay calibration of change in absorbance at 365 nm to BY-2 extract concentration.

Reduction of APAD in the coupled citrate synthase assay was detected as a linear increase in absorbance at 365 nm. The region of linearity corresponded to APAD (and acetyl-CoA) consumption up to 4.1 mM min⁻¹. Data points = mean ± SEM, n = 3. R² for all replicates = 0.994, y = 0.000125x – 0.000663.

[3-¹⁴C]-HMG-CoA) and at R_f 0.6 (*S*-[3-¹⁴C]-mevalonate). Radioactivity was initially measured by phosphorimaging. Radioactivity of the mevalonate fraction increased linearly with microsomal extract up to $25.6\% \pm 1.2\%$ HMG-CoA consumption (mean $\pm$ SEM, $n = 3$; Figure 3.14. An example of a scanned HMGR assay phosphorimaging plate is presented in Appendix 3.5).

It was noticed that phosphorimaging results varied with exposure. Experimental replicates were performed of phosphorimaging, TLC and the assay to determine the cause of this variation. Coefficients of variation were 0.22 ± 0.06 , 0.09 ± 0.02 and 0.10 , respectively. Phosphorimaging accuracy was subsequently verified by scintillation counting of chromatography plate scrapings. This gave similar results to phosphorimaging, with linearity up to $25.8\% \pm 1.0\%$ HMG-CoA consumption (mean $\pm$ SEM, $n = 3$; Figure 3.14). Two-way analysis of variance revealed no significant differences between the two methods of analysis.

WT BY-2 HMGR activity in microsomal extracts from cultures at four days was determined by phosphorimaging to be 351 ± 30 pmol min⁻¹ (mg protein)⁻¹ and by scintillation counting to be 381 ± 43 pmol min⁻¹ (mg protein)⁻¹ (mean $\pm$ SEM, $n = 14$).

3.3. Discussion

The results in this chapter established a suitable medium for MFA of BY-2 cells, validated the metabolite and enzyme assay methods used in subsequent analyses of manipulations to metabolism, and provide information about the development of WT BY-2 cultures.

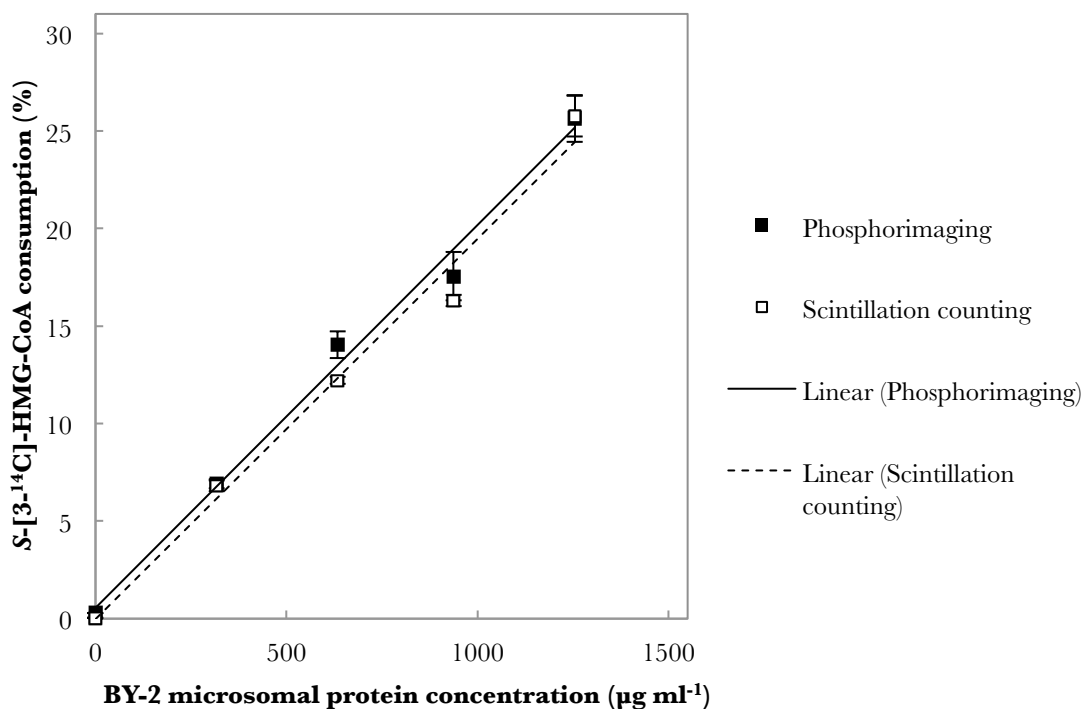


Figure 3.14. Linearity of HMGR assay with respect to wild-type BY-2 microsomal extract protein concentration.

Conversion of *S*-[3-¹⁴C]-HMG-CoA to *S*-[3-¹⁴C]-mevalonate was measured in WT BY-2 microsomal extracts using a stopped assay. Assay products were separated by thin layer chromatography. Chromatography plates were then analysed by phosphorimaging and scintillation counting of plate scrapings. There were no significant differences between the two analytical methods (two-way ANOVA). Linear regression results were as follows: $y = 0.00020x + 0.0062$, $R^2 = 0.969$ for all phosphorimaging replicates; $y = 0.00020x - 0.0004$, $R^2 = 0.974$ for all scintillation counting replicates. Data points represent mean $\pm$ SEM, $n = 3$.

3.3.1. 2% glucose medium is optimal for the study of BY-2 metabolism

The suspension culture growth profiles for WT BY-2 compared favourably with published data (Hatzfeld et al., 1998; Hemmerlin and Bach, 1998; Wentzinger et al., 2002). The preference for 2% glucose medium over 3% glucose medium was also consistent with published observations (Disch et al., 1998). This suggested that 2% glucose medium would be optimal for metabolic flux analysis and led to the adoption of 2% glucose medium for subsequent experiments.

The variability of DW to FW measurements from aliquots of cultures younger than three days is probably indicative of the high degree of error in measuring very small masses, while the variability of DW to FW ratios of cultures of the same age harvested in their entirety was probably due to the difficulty in adequately harvesting the entire culture (with some cells left in the flask or on the filtration equipment). The consistent finding that the DW to FW ratio decreased with increasing culture density contradicts more in-depth investigations of BY-2 culture growth, which found that the vacuole and cytoplasm quickly accumulate phosphate after subculture, resulting in the uptake of water (Nagata et al., 1992). In the later logarithmic phase, phosphate is exported from the vacuole to replenish the depleting supplies in the cytosol, with the result that cells lose water and become smaller (Nagata et al., 1992). Thus, it is likely that the observed differences in the DW to FW ratio result from an increasing difficulty in removing the culture medium as the population density increases.

3.3.2. GC-MS is the preferred method for sterol identification and quantification

The lack of consistency in published sterol ^1H -NMR chemical shifts, along with the complexity of the mixtures being analysed, made for an unreliable process of compound identification when analysing sterol extracts by ^1H -NMR. Prior separation of hexane extracts, for instance by TLC, and verification of standard compounds might have allowed identification of individual sterols, but this was not desirable when identification by GC-MS proved straight-forward and reliable, and when NMR spectroscopy was more labour-intensive and time-consuming.

Due to the variety of sterols in the BY-2 hexane extracts, it was desirable to pool the response data to individual sterols to achieve a single quantification formula for all sterols. This was done, but the formula is only accurate if the GC-MS detector is similarly sensitive to all sterols. The data from the four sterols of the Matreya plant sterol mix suggest this to be so, but these sterols are all 4-desmethyl Δ^5 sterols with C-24 alkyl groups. Sterols are quite a structurally diverse group. For example, cycloartenol differs from the four sterols of the Matreya plant sterol mix by being dimethylated at C-4, lacking a C-24 alkyl group, having a saturated ring structure, and containing a $9\beta,19$ -cyclopropane ring. It is possible that GC-MS detector sensitivity to structurally different sterols, which primarily are upstream pathway intermediates, differs from the sensitivity to the sterols used in the calibration experiments.

Similarly, it is possible that upstream pathway intermediates are less stable than the sterols assessed for recovery by alkaline saponification and hexane extraction. There is a dearth of data available on either the intrinsic stability of individual sterols or their stability during extraction. The only relevant study indicates that sitosterol is slightly less stable during saponification than cholesterol (Busch and King, 2010). The wide variety of sterols isolated

from BY-2 cells made it desirable to accept an overall recovery value. Although the relatively low recovery of brassicasterol was initially troubling, brassicasterol appears to be peculiar to the Brassicaceae (Benveniste, 2002). It was not detected in BY-2 extracts presented here. Neither are there published examples of brassicasterol in sterol extracts from other tobacco sources (Gondet et al., 1994; Schaller et al., 1995; Hartmann and Bach, 2001; Holmberg et al., 2002; Hemmerlin et al., 2003; Holmberg et al., 2003; Hemmerlin et al., 2004; Hey et al., 2006; Roche et al., 2008). As such, and with average sterol recovery being approximately 100%, it seemed reasonable to accept the assumption that recovery of BY-2 sterols was 100%.

3.3.3. The BY-2 sterol profile varies with culture age

Total BY-2 sterol content of 1.5-1.8 ng (mg DW)⁻¹ is similar to a published value of 1.9 ng (mg DW)⁻¹ (Wentzinger et al., 2002). The proportions of individual sterols were also in close agreement with published values for BY-2 cells (Wentzinger et al., 2002; Mongrand et al., 2004; Roche et al., 2008), further validating the identification and quantification methods. Stigmasterol, sitosterol, campesterol, cholesterol and cycloartenol are known to increase lipid order to some degree, and it is likely that other sterols do too (Schuler et al., 1990; Schuler et al., 1991; Krajewski-Bertrand et al., 1992). Sitosterol and campesterol, which are the most abundant sterols at four days and seven days, are the most efficient sterols at decreasing membrane permeability to H₂O and membrane fluidity, whereas stigmasterol has no effect on membrane permeability and very little on lipid order (Schuler et al., 1990; Schuler et al., 1991; Krajewski-Bertrand et al., 1992). Additionally, sitosterol and campesterol inhibit membrane-bound H⁺-ATPase, whereas stigmasterol (as well as cholesterol) appears to cause proton efflux by stimulating H⁺-ATPase (Grandmougin-Ferjani et al., 1997).

Cultures at four days had a higher proportion of intermediate sterols than at seven days. The differences in sterol profiles at these two time points may reflect changes in requirements for cell membrane components. During log phase there is a high demand for sterols as part of membrane biogenesis, and the sterol profile may represent the pathway at a productive metabolic steady state. During stationary phase, when less membrane biogenesis is occurring, less sterol biosynthesis is likely to be required, and the profile may represent this pathway close to stasis.

3.3.4. Citrate synthase activity and levels of acetyl-CoA and capsidiol were as expected

CS activity in the BY-2 WT was comparable to tomato leaf (Nunes-Nesi et al., 2007; Sienkiewicz-Porzucek et al., 2008; Sulpice et al., 2010) and tobacco leaf (Scheible et al., 2000) extracts, but was about 10-20% lower than CS activity measured in *Arabidopsis* (Koyama et al., 2000), carrot cell culture (Koyama et al., 1999), canola leaf (Anoop et al., 2003) and tobacco root (Delhaize et al., 2001; Delhaize et al., 2003) extracts.

Acetyl-CoA content of approximately 2 pmol (mg FW)⁻¹ in the WT was comparable to 0.6 to 30 pmol (mg FW)⁻¹ in various plant tissues (Geigenberger et al., 2000; Bender-Machado et al., 2004; Tumaney et al., 2004; Vigeolas et al., 2007; Usadel et al., 2008; Perera et al., 2009). The absence of capsidiol in CHCl₃ extracts is in agreement with published data on unelicited tobacco KY-14 cell (Chappell and Nable, 1987; Chappell et al., 1989; Chappell et al., 1991) and hairy root cultures (Wibberley et al., 1994), although tobacco var. *xanthi* cultures secreted up to 4 µg ml⁻¹ capsidiol into the culture medium (Milat et al., 1991). The results suggest that

capsidiol is not synthesised (or is synthesised at exceedingly low levels) by WT BY-2 cells grown under standard conditions.

3.3.5. Metabolome profiling reveals changes in primary metabolite composition as cultures progress from logarithmic to stationary growth

There were difficulties associated with the TagFinder-based approach to metabolome profiling. One difficulty was in distinguishing between different compounds with the same mass spectra that elute sequentially. The failure of time groups to consistently align across all samples was another problem. Despite these issues and the consequent need for some manual curation of results, the results presented here demonstrate that this GC-MS-based metabolomics approach is sensitive enough to reveal differences in the abundances of individual analytes between transgenic lines. However, abundance values for each analyte varied widely within each group. This suggests that more replicates are desirable in order to make statistically robust conclusions on the relative levels of individual metabolites. This is especially relevant as Bonferroni correcting for multiple comparisons increases the stringency of the critical P-value and, therefore, the likelihood of encountering type II statistical errors (Perneger, 1998), where real differences between samples in different experimental conditions are missed. PCA coupled with MANOVA provides a more robust method of determining if metabolite profiles of experimental groups differ.

β -alanine, threonate and ethanolamine were the only metabolites found to differ significantly over the course of the experiment. β -alanine levels approximately doubled as cultures aged from five to six days. β -alanine levels in various plant species have been observed to increase following heat-shock (Mayer et al., 1990; Kaplan et al., 2004) and growth under high salinity

(Hanson et al., 1991) and drought conditions (Rizhsky et al., 2004). It seems feasible that the observed increase in abundance in β -alanine between five and six days is part of a stress response to a possible depletion of glucose in the culture medium.

Threonate levels dropped by about 60% between four and six days. Threonate is a degradation product of ascorbate (Green and Fry, 2005). Although ascorbate was not detected by these methods, ascorbate content of BY-2 cells reportedly decreases as growth rate decreases, and high levels of ascorbate may even stimulate cell division (de Pinto et al., 1999). It is possible that the reduction seen in this metabolite is a direct result of reduced levels of ascorbate as population growth slows. However, it should be noted that threonate more typically accumulates as ascorbate is degraded (Loewus, 1999). Also, there were no changes in the levels of other possible products of ascorbate degradation, such as dehydroascorbate or erythronate.

Ethanolamine was observed to accumulate towards the stationary growth phase. Ethanolamine is synthesised primarily by decarboxylation of serine (Mudd and Datko, 1989; Rontein et al., 2001; Rontein et al., 2003), and it is noticeable that mean levels of each of the three serine-TMS ethers (2-, 3- and 4TMS) were also increased at six days, with two and three times the contents at five and four day levels, respectively (Appendix 3.3). Ethanolamine is an intermediate in phospholipid biosynthesis, and the accumulation could be the result of decreased phospholipid biosynthesis as growth slows. However, this would seem to indicate an unexpected lack of coordination between ethanolamine biosynthesis and phospholipid biosynthesis. Ethanolamine is also a product of *N*-acylethanolamine hydrolysis (Chapman and Blancaflor, 2010). *N*-acylethanolamines are synthesised endogenously by tobacco KY-14 cells (Chapman et al., 1995; Chapman et al., 1998) and can stimulate growth in some plant systems (Han et al., 2007). An alternative explanation for the increased ethanolamine levels is that *N*-acylethanolamines are hydrolysed to slow growth as glucose is depleted from the medium.

The results of multivariate statistical analyses indicate that the soluble metabolite profile changes as culture growth slows (days five to six, and, more strikingly, between days four and six). However, metabolome changes were slight, with single principal components providing resolution of culture age only becoming apparent when pairs of ages were compared. The similarity of the profiles at days four and five implies that the cultures are at a metabolic steady state during logarithmic growth. Although PCA provides the loadings for each analyte with respect to each principal component, it is not statistically robust to consider levels of analytes with non-zero loadings to have changed except for when univariate statistical analyses support this conclusion. Nevertheless, the results suggest that, as growth slows, the proportions of sterols and amino acids increase. However, it should be understood that these are changes in the levels of individual metabolites in groups. Due to the scaling used in PCA and the lack of absolute quantification, these trends may not be indicative of changes in the absolute levels of these metabolite groups.

There is an apparent general increase in the concentrations of methanol-soluble metabolites as growth progresses from log-phase to stationary-phase. This may be due to the utilisation of starch as glucose in the culture medium is depleted (Krook et al., 1998). If so, this finding complicates comparisons of metabolite levels normalised to total biomass at different growth phases.

3.3.6. HMGR activity can be assayed, but absolute values may be unreliable

HMGR spectrophotometric assay results indicated that this assay was insufficiently sensitive to measure HMGR activity in BY-2 total and microsomal protein extracts. Although there was a high degree of variability in phosphorimaging of ^{14}C HMGR assay results, the agreement

between scintillation counting results and averaged phosphorimaging results indicated that the variation in phosphorimaging could be overcome by performing multiple plate exposures. As such, phosphorimaging of ^{14}C -HMGR assay plates was adopted as the analytical method of choice, with an average value from three separate exposures used to calculate enzyme activity.

HMGR activity of approximately $350 \text{ pmol min}^{-1} (\text{mg protein})^{-1}$ compares to published values for BY-2 microsomal extracts from mid log-phase growth of $12\text{-}160 \text{ pmol min}^{-1} (\text{mg protein})^{-1}$ (Hemmerlin and Bach, 1998; Hemmerlin and Bach, 2000; Hemmerlin et al., 2003). Thus, the values measured here are slightly higher than expected, although the wide range of published values may indicate the high degree of variability inherent in this assay. This variability is particularly relevant when comparing different transgenic lines or treatments and may be partly due to variable microsomal extract quality. To deal with this potential variability, it was decided that lines should be compared within extraction batches, with multiple extraction batches performed to provide statistical robustness.

3.3.7. Summary

The results presented here indicate that the WT BY-2 line was healthy and grew well in medium containing glucose, the preferred substrate for the MFA presented in Chapter 5. Assays for which quantification was desired produced linear responses with low variation between replicates. Analyses of targeted metabolites (sterols, acetyl-CoA and capsidiol) yielded results that were in good agreement with published results either from BY-2 cultures or from other plant systems. Similarly, the results of the CS assay were in approximate agreement with published plant values. HMGR activity values presented here and published values vary widely due largely to the high degree of variability inherent in the assay. Recommendations

were made for dealing with this variability. Similarly, there is a high degree of statistical error in results of the metabolomics study, and recommendations were made for dealing with this and with potential analytical errors that arise from using the TagFinder software.

Chapter 4. Increasing the supply of precursors for sterol biosynthesis by increasing flux from mitochondrial acetyl-CoA to cytosolic acetyl-CoA

4.1. Introduction

4.1.1. Objectives

The aims of this chapter were:

1. to identify an enzyme at the interface of primary metabolism and sterol biosynthesis that could be manipulated to increase the availability of cytosolic acetyl coenzyme A (acetyl-CoA) for sterol biosynthesis in tobacco (*Nicotiana tabacum*) Bright Yellow 2 (BY-2) cells;
2. to establish the effect of such a manipulation on steady state levels of acetyl-CoA, citrate and sterols in BY-2 cells;
3. to study the metabolic phenotype resulting from this manipulation in more detail.

4.1.2. Potential targets for increasing cytosolic acetyl-CoA availability

As previously discussed, sterols are derived from cytosolic acetyl-CoA. This is exported from the mitochondrial tricarboxylic acid (TCA) cycle in the form of citrate (Figure 4.1). Various enzymes and transport proteins involved in the production of cytosolic acetyl-CoA, beginning with the decarboxylation of pyruvate to form mitochondrial acetyl-CoA, were considered as

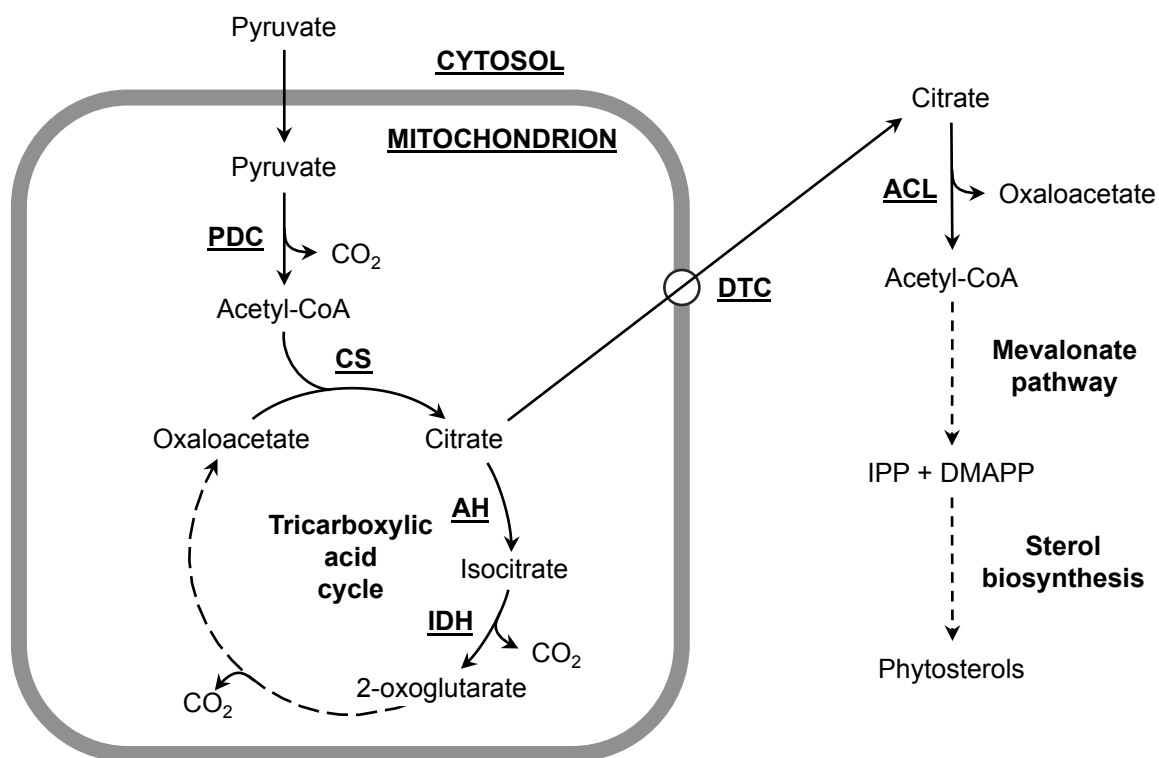


Figure 4.1. Metabolic pathway diagram illustrating the link between sterol biosynthesis and primary metabolism.

Dashed arrows represent several serial reactions. Only steps discussed in the main text are shown in full. Competing reactions are not shown.

Abbreviations: **ACL**, ATP-citrate lyase; **AH**, aconitase; **CS**, citrate synthase; **DMAPP**, dimethylallyl diphosphate; **DTC**, dicarboxylate-tricarboxylate transporter; **IDH**, isocitrate dehydrogenase; **IPP**, isopentenyl diphosphate; **PDC**, pyruvate dehydrogenase complex.

potential targets for increasing the availability of cytosolic acetyl-CoA. Essentially two approaches were considered with respect to this: either increasing the levels of enzymes or carrier proteins directly necessary for the conversion of mitochondrial pyruvate to cytosolic acetyl-CoA, or decreasing the activity of enzymes in competing metabolic pathways.

Pyruvate dehydrogenase

The mitochondrial pyruvate dehydrogenase complex catalyses the decarboxylation of pyruvate to form acetyl-CoA while reducing NAD⁺. Pyruvate dehydrogenase is a multienzyme complex, which complicates any attempts at increasing activity by gene over-expression. However, the mitochondrial complex is negatively regulated by pyruvate dehydrogenase kinase. Antisense suppression of pyruvate dehydrogenase kinase in *Arabidopsis* (*Arabidopsis thaliana*) plants resulted in increased extractable activity of pyruvate dehydrogenase and citrate synthase (CS) (Zou et al., 1999; Marillia et al., 2003). The effects of these changes on the metabolome and the fluxome were not specifically investigated. However, feeding with [¹⁴C]pyruvate did result in greater labelling of fatty acids in antisense lines than control lines, presumably due to increased flux through mitochondrial acetyl-CoA and increased export of citrate to the cytosol (Marillia et al., 2003).

Citrate synthase

CS catalyses the condensation of acetyl-CoA and oxaloacetate to form citrate and CoA-SH. Much research has been conducted into the effect of citrate efflux from plant roots as a means of increasing a plant's tolerance to Al³⁺ and Zn²⁺ in soil through the chelation of metal cations. Sugar (*Beta vulgaris*) beet grown in high Zn²⁺ conditions accumulated citrate at increased levels (Sagardoy et al., 2011). Increased CS activity and decreased isocitrate dehydrogenase were associated with this increase in citrate content. Similarly, Al³⁺ stimulated efflux of citrate from *Senna tora* roots, and this was accompanied by increased CS activity and reduced aconitase activity (Yang et al., 2004). In neither of these studies was the sub-cellular

location of the increases in CS activity determined. However, over-expression of mitochondrial CS in carrot (*Daucus carota*) cells resulted in a doubling of CS protein and a tripling of citrate excretion when cells were grown on insoluble phosphate medium (Koyama et al., 1999). Citrate excretion was also enhanced following over-expression of a mitochondrial CS in Arabidopsis (Koyama et al., 2000) and oilseed rape (*Brassica napus*) (Anoop et al., 2003) subjected to phosphorous limitation and aluminium toxicity, respectively.

Research has also been conducted into the role of CS in the accumulation of citrate in fruit during ripening. In some species citrate accumulation is associated with increased CS activity (Iannetta et al., 2004; Saradhulhat and Paull, 2007), although this is not always the case (Etienne et al., 2002; Tang et al., 2010), and the process of vacuolar citrate accumulation during ripening involves changes in the activities of several enzymes involved in citrate biosynthesis and degradation (Etienne et al., 2002; Tang et al., 2010; Morgan et al., 2013). Finally, a further study on tomato (*Solanum lycopersicum*) has revealed that leaves with reduced mitochondrial CS have decreased flux through mitochondrial CS (Sienkiewicz-Porzucek et al., 2008).

Dicarboxylate-tricarboxylate carrier

Dicarboxylate-tricarboxylate carrier (DTC) proteins exchange organic acids, including citrate (in the H-citrate²⁻ form), across the mitochondrial membrane (Picault et al., 2002). At least four different genes encoding transport proteins with affinity for citrate have been identified in tobacco (Picault et al., 2002). There are no published attempts at over-expressing DTCs. However, a correlation between malate content and the expression levels of two DTC genes in ripening grape (*Vitis vinifera*) berries has been identified (Regalado et al., 2013). The authors inferred that a fall in malate content was due, in part, to increased DTC expression by a mechanism that involves transport of malate from the vacuole to the mitochondrion, where it is metabolised by malate dehydrogenase. The evidence they present for this is weak, but if the

DTC does exercise significant control over malate flux across the mitochondrial membrane in grapes, it may do the same for citrate in other systems. DTC expression in *Citrus junos* seedlings is correlated with Al^{3+} content of the growth medium (Deng et al., 2008). This, along with the correlation seen elsewhere between Al^{3+} content of the growth medium and citrate efflux, suggests that some regulation of organic acid export from mitochondria is provided by DTCs.

ATP-citrate lyase

ATP-citrate lyase (ACL) catalyses the conversion of citrate to acetyl-CoA and oxaloacetate. Plant ATP citrate lyase is an heterooctomer comprising ACLA and ACLB subunits (Fatland et al., 2000). Both of these subunits are required for function and are each encoded by multiple genes, with the products of at least *ACLA1* and *ACLB2* being located in the cytosol (Fatland et al., 2002).

ACL has frequently been investigated in cancer research. Reduced levels of ACL in mammalian cells reduced histone acetylation (Wellen et al., 2009) and lipid biosynthesis (Hatzivassiliou et al., 2005). The reduced lipid biosynthesis resulted in cell cycle arrest (Zaidi et al., 2012). However, this reveals little other than reinforcing the link between cytosolic citrate and lipid biosynthesis in mammals. A similar phenotype was found following antisense suppression of *ACLA* in *A. thaliana* (Fatland et al., 2005). Plants with less than half of wild-type (WT) levels of cytosolic ACL suffered from dwarfism in addition to reduced fatty acid and flavonoid production, suggesting a significant restriction in flux into cytosolic acetyl-CoA (Fatland et al., 2000; Fatland et al., 2005).

Aconitase

Aconitase interconverts citrate and isocitrate in the TCA cycle and in the cytosol. Sour lemon (*Citrus limon*) fruit exhibit a reduction in mitochondrial aconitase activity as citrate starts to

accumulate (Sadka et al., 2000; Degu et al., 2011). Similarly, mutant tomato fruit with increased vacuolar citrate content had decreased cytosolic aconitase activity (Morgan et al., 2013). Mutant tomato plants with reduced cytosolic and mitochondrial aconitase activity had higher citrate content in leaves, but these plants also had a slow growth phenotype (Carrari et al., 2003). As already discussed, *S. tora* plants grown at high Al^{3+} concentrations had increased citrate efflux associated with reduced aconitase activity (as well as increased CS activity) (Yang et al., 2004). These results suggest that reducing demand from the TCA cycle for citrate causes some accumulation of citrate, probably in the vacuole.

Isocitrate dehydrogenase

Isocitrate dehydrogenase catalyses the oxidative decarboxylation of isocitrate to α -ketoglutarate. Antisense suppression of isocitrate dehydrogenase in tobacco plants led to increased shoot and root citrate content (Delhaize et al., 2003). As already discussed, decreased isocitrate dehydrogenase activity was associated with increased citrate content in sugar beet grown in high Zn^{2+} medium (Sagardoy et al., 2011), but larger changes in CS activity were discovered under these conditions.

4.1.3. Citrate synthase is a promising candidate for increasing cytosolic acetyl-CoA availability

Choosing a suitable candidate for genetic manipulation is complicated by the wide variety of organisms on which treatments have been tested: an effect of a manipulation in one organism may not translate into a similar effect in another organism, and so it is difficult to compare the

manipulations. However, undesirable characteristics were apparent in manipulations of most of the candidates.

The pyruvate dehydrogenase complex was rejected due to the lack of a suitable method of establishing changes in pyruvate dehydrogenase activity. There is also a lack of published characterisation of the effects of reduced pyruvate dehydrogenase activity on cytosolic acetyl-CoA or sterol formation in the absence of artificially high pyruvate concentrations.

The DTC was rejected due to the lack of any direct evidence of alterations in DTC levels having an effect on cell citrate content. Assaying transport activity across the mitochondrial membrane between different transgenic lines was also expected to be problematic.

Mammalian ACL is a homotetramer encoded by a single gene product (Sato et al., 2000), and overexpression of this gene would circumvent the difficulties associated with co-expression of two plant ACL genes. However, mammalian ACL is characterised by complex post-transcriptional and post-translational modifications (Sato et al., 2000). Additionally, while it is likely that small reductions in ACL activity restrict flux into cytosolic acetyl-CoA (Fatland et al., 2000; Fatland et al., 2005), it is less clear if increased ACL activity would be likely to significantly affect that flux.

Antisense suppression of aconitase was rejected because the reduced TCA cycle flux negatively impacted on other aspects of metabolism in tomato plants with reduced aconitase (Carrari et al., 2003), and the reduced aconitase activity apparent in sour lemons was measured in juice sac cells (Sadka et al., 2000), which were not dividing. Antisense suppression of isocitrate dehydrogenase was similarly unfavourable because of the negative effects that reduced TCA cycle flux could have on culture growth.

Over-expression of mitochondrial CS in plants has proven efficacy for increased citrate synthesis (Koyama et al., 1999; Koyama et al., 2000); it is a monomeric protein, which allows over-expression to be conducted relatively straightforwardly; and CS activity in protein extracts can be assayed quickly and reliably, which allows for validation of many transgenic lines. These features were not shared by any of the other candidate proteins, which either had a lack of established efficacy at increasing cytosolic citrate or acetyl-CoA levels during normal growth regimes (ACL, DTC and pyruvate dehydrogenase), are difficult to assay (DTC) or require antisense suppression, which was deemed more likely to cause undesirable pleiotropic effects (aconitase and isocitrate dehydrogenase). For these reasons, CS was over-expressed in BY-2 cells. The effects of this over-expression were characterised with respect to extractable CS activity, growth, O₂ uptake and the cytoplasmic levels of citrate, acetyl-CoA, sterols, and the metabolome more generally.

4.2. Results

4.2.1. Generation of *ATCS* and control expression vectors

The pH7WG2D vector (Karimi et al., 2002) was chosen for expression of Arabidopsis *CITRATE SYNTHASE 4* (*ATCS*; At2g44350), which encodes a mitochondrion-targeted CS. This was primarily because it contains the cauliflower mosaic virus 35S promoter, which provides high-level constitutive expression, and because it confers hygromycin resistance, which would facilitate co-transformation with a 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) vector described in Chapter 6. The pH7WG2D transfer DNA (T-DNA) also contains a green fluorescent protein (GFP) gene, allowing straightforward epifluorescence

screening of hygromycin-resistant calli before performing biochemical assays, and transgenes can be easily inserted into the vector using Gateway recombination.

The open reading frame of *ATCS* was amplified from an Arabidopsis cDNA library. *mCherry* (Shaner et al., 2004) was used as a control transgene. The *mCherry* coding sequence was amplified from pRSET-B (kindly provided by Dr Monika Kalde). *ATCS* and *mCherry* entry clones were generated by T/A cloning of the amplified coding sequences into pCR8/GW/TOPO. Clones containing inserts in the entry vector in the correct orientation were identified by polymerase chain reaction (PCR). Gateway cloning transferred inserts to pH7WGD via pDEST42 and pDONR201. These two intermediate vectors were used because pCR8/GW/TOPO and pH7WG2D each confer spectinomycin resistance in *E. coli*. pDEST42 and pDONR201, by contrast, confer resistance to ampicillin and kanamycin, respectively. Success after each Gateway reaction and transformation was confirmed by transgene PCR using antibiotic-resistant *E. coli* colonies and purified plasmids as templates (Figure 4.2), and by restriction digestion of purified plasmids. Inserted transgenes in the pH7WG2D-*mCherry* and pH7WG2D-*ATCS* expression vectors were confirmed by sequencing. Construct maps of *mCherry* and *ATCS* T-DNAs are presented in Figure 4.3. The DNA sequences of pH7WG2D-*mCherry* and pH7WG2D-*ATCS* are presented in Appendices 4.1 and 4.2, respectively.

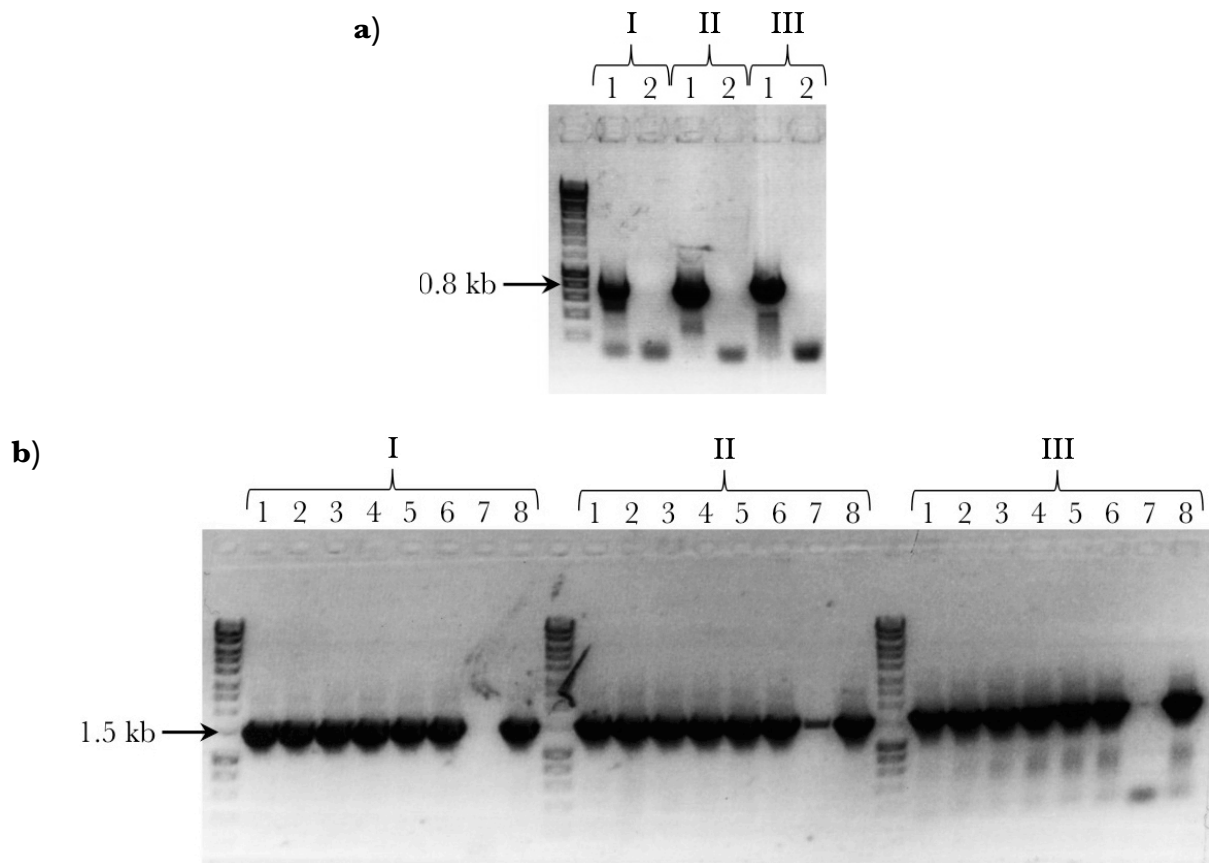


Figure 4.2. PCR analysis of control and *ATCS* expression vectors.

a) PCR amplification of *mCherry* from the pH7WG2D-*mCherry* Gateway expression vector. Primer pairs were I: mCherry-F/mCherry-R, which anneal the 5' and 3' ends of the *mCherry* gene, respectively (0.7 kb expected product); II: mCherry-F/p7WG2-ins-R, the latter of which anneals immediately downstream of the insert site of the pH7WG2D vector (0.9 kb expected product); III: p7WG2-ins-F/mCherry-R, the former of which anneals immediately upstream of the insert site of the pH7WGD vector (0.8 kb expected product). Lane 2 of each primer pair is a PCR negative control with H₂O as template. Lane 1 is a 1:100 dilution of a plasmid preparation in H₂O. **b)** PCR of *ATCS* cDNA from the pH7WG2D-*ATCS* Gateway expression vector. Primer pairs were I: CS-OX-F/CS-OX-R, which anneal to the 5' and 3' ends of the *ATCS* cDNA, respectively (1.5 kb expected product); II: CS-OX-F/p7WG2-ins-R (1.5 kb expected product); III: p7WG2-ins-F/CS-OX-R (1.6 kb expected product). Lanes 1-6 of each primer pairing are 1:100 plasmid preparations in H₂O. Lane 7 is an H₂O negative control. Lane 8 is a positive control using an earlier preparation of pK7WG2D-*ATCS*, which confers resistance to kanamycin rather than hygromycin. DNA markers in each gel were HyperLadder I (Bioline Reagents Ltd).

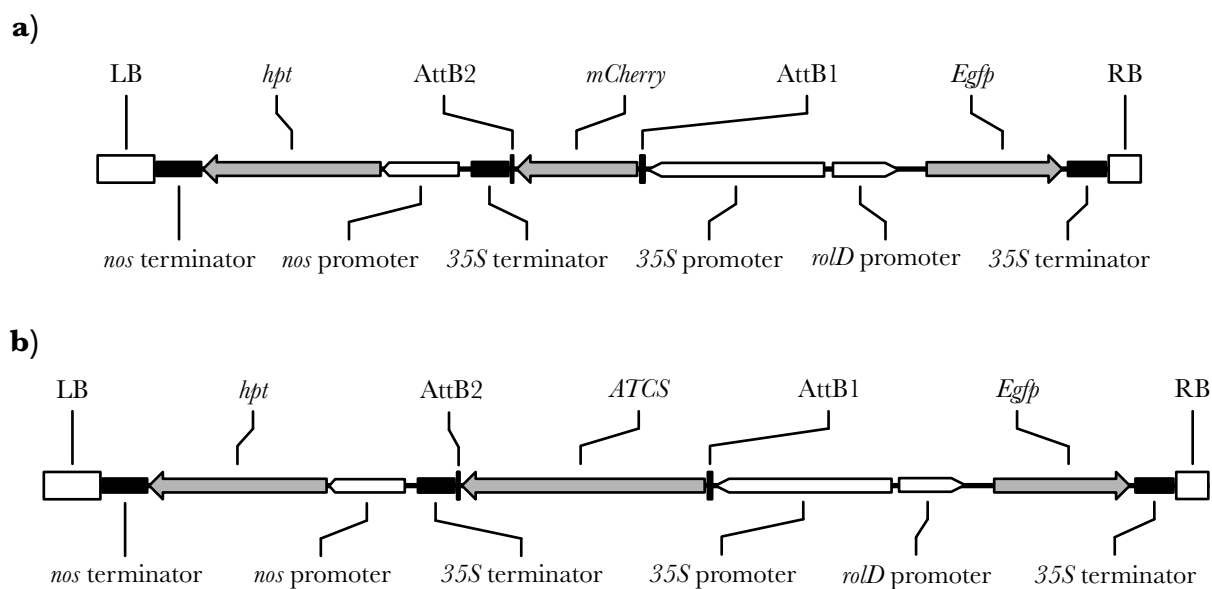


Figure 4.3. Overview of *ATCS* and control transfer DNA segments.

Expression vectors were synthesised by Gateway recombination of transgenes into the pH7WG2D destination vector (Karimi et al., 2002). **a)** *mCherry* (control) construct (6.1 kb). **b)** *ATCS* construct (6.9 kb).

Abbreviations: **AttB1** and **AttB2**, Gateway recombination sites; *Egfp*, enhanced GFP; *hpt*, hygromycin phosphotransferase; **LB** and **RB**, T-DNA borders.

4.2.2. Screening of hygromycin-resistant BY-2 for increased citrate synthase activity

ATCS transformation of BY-2 cells yielded 53 hygromycin-resistant calli. A smaller volume of cells was plated for the control transformation, which yielded nine hygromycin-resistant calli. Prior to assaying CS activity, a subset of calli was screened for GFP (*ATCS* and control) and mCherry (control) fluorescence to provide additional evidence of transformation success (Appendix 4.3). All screened antibiotic resistant calli exhibited GFP fluorescence, and control lines exhibited mCherry fluorescence. Untransformed WT calli did not fluoresce significantly.

18 *ATCS* lines and three control lines were lost after the initial transfer of transgenic calli to fresh solid medium due to contamination. The remaining 35 *ATCS* and six control calli were screened for increased CS activity (Figure 4.4a). Extracts from *ATCS* calli exhibited an average CS activity 2.7 times that of controls ($p < 0.01$). CS activity in 25 *ATCS* calli extracts was higher than any assayed control line. CS activity in *ATCS* line extracts varied from 13-140 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. The range for control extracts was less variable at 8-30 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$.

The five *ATCS* lines exhibiting highest CS activity in callus protein extracts (lines 6, 16, 34, 45 and 49) and three control lines (4, 5 and 6) were taken into suspension medium. These went through two rounds of subculture before four-day-old cultures were assayed for CS activity (Figure 4.4b). Extracts from all *ATCS* lines, except for line 45, had higher CS activity than any of the control lines ($p < 0.05$). The highest CS activity, in *ATCS* lines 6 and 16, was 2.4 times the average of the controls.

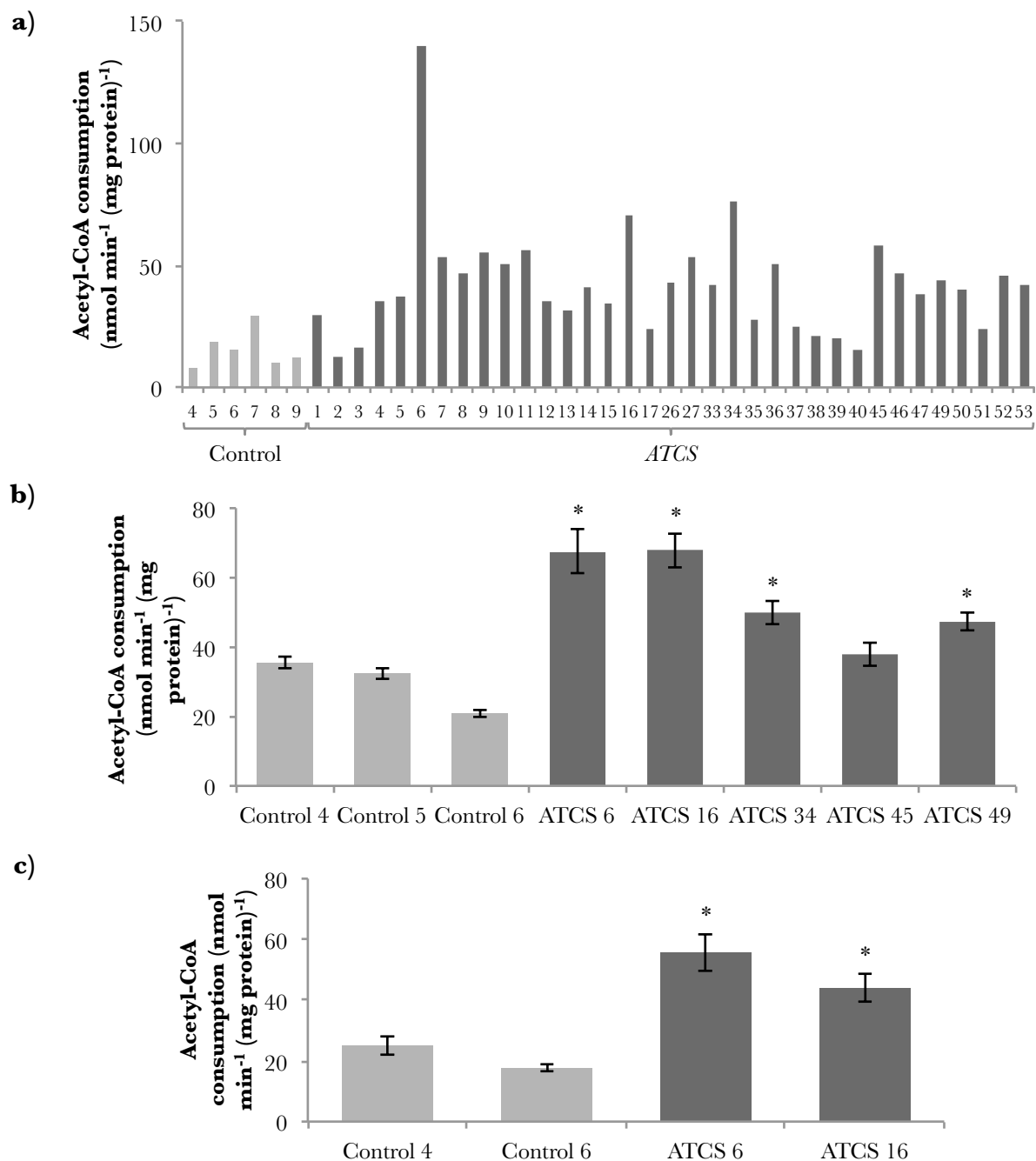


Figure 4.4. Citrate synthase activity of transgenic *ATCS* BY-2 lines.

a) CS activity of hygromycin-resistant calli following transformation with either the control construct or the *ATCS* construct. **b)** Three control calli and the five *ATCS* calli with highest CS activity were grown in suspension, and CS activity from four-day-old cultures was measured (n = 3). **c)** CS activity in the two *ATCS* lines exhibiting highest activity in (b) and two control lines was measured periodically during log-phase (n = 9). This represents the average CS activity over the course of 293 days. CS activity measured in each assay is presented in Appendix 4.4. “*” denotes that the measurement is significantly different to measurements of each of the control lines (p < 0.05, student’s *t* test). Error bars represent standard error (SEM).

ATCS lines 6 and 16, and the two control lines with highest and lowest CS activity (4 and 6, respectively), were maintained in suspension culture for further analyses. CS activity was assayed periodically to check that CS activity remained consistent through successive subculture cycles (Appendix 4.4). *ATCS* lines 6 and 16 had consistently increased CS activity compared to the controls (Figure 4.4c). *ATCS* line 6 CS activity was 2.6 ± 0.2 times the control average (mean $\pm$ SEM, $n = 9$, $p < 0.0005$). *ATCS* line 16 CS activity was 2.1 ± 0.1 times that of controls ($p < 0.0005$). *ATCS* line 6 had significantly higher CS activity than *ATCS* line 16 ($P < 0.05$), with an average activity 1.3 ± 0.1 times higher.

4.2.3. Growth analysis of *ATCS* and control lines

ATCS lines were initially analysed to determine if growth rate had been affected by the transformations, which might indicate altered carbon conversion efficiency or respiration. Fresh weight (FW) of *ATCS* lines 6 and 16, and control lines 4 and 6, was measured at 24 h intervals (Figure 4.5). *ATCS* line 16 had similar growth dynamics to the two control lines, with logarithmic growth occurring between two days and five to six days after subculture. *ATCS* line 6 grew more slowly, with an extended logarithmic phase between two and seven days. This was evidenced by FW being significantly less than each of the other three lines at days four, five and six ($p < 0.05$). FW of *ATCS* line 6 reached control levels by approximately seven days.

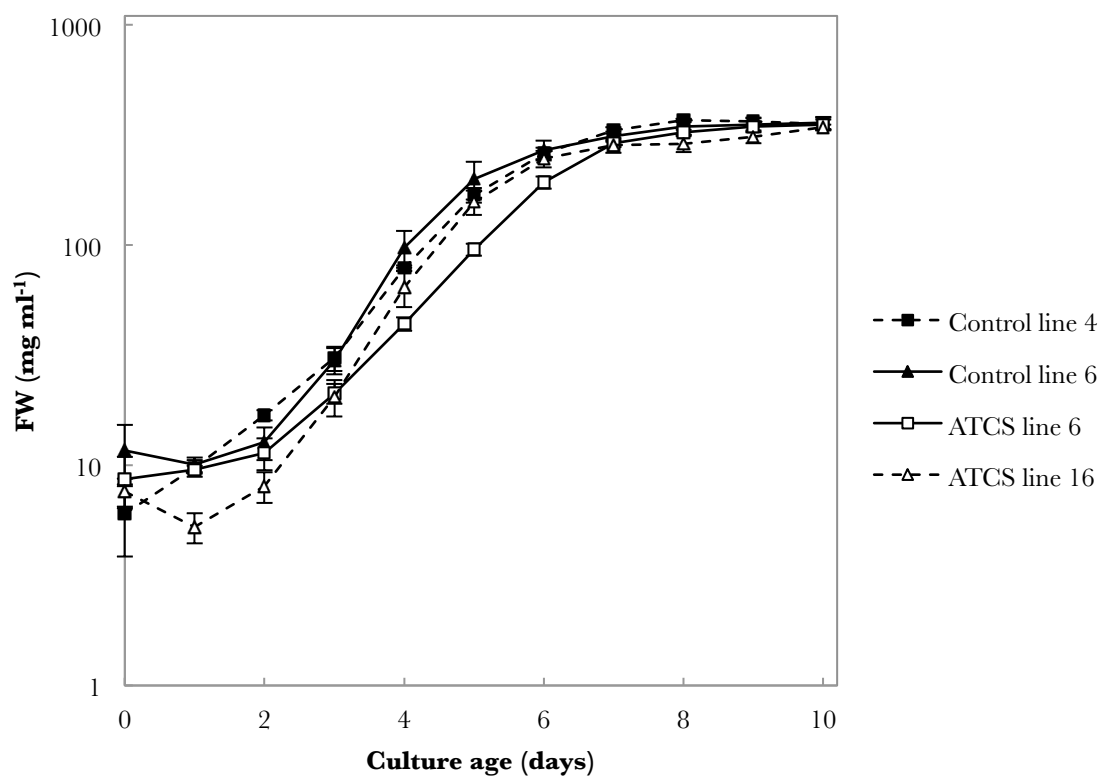


Figure 4.5. Growth analysis of transgenic *ATCS* and control line BY-2 cell cultures.

FW of aliquots from transgenic suspension cultures was measured at 24 h intervals. Data points represent mean $\pm$ SEM, n = 4.

4.2.4. Oxygen uptake measurements of *ATCS* and control lines

It was considered that increased CS activity might result in increased respiratory flux, and that this would be evident as increased O₂ uptake by the *ATCS* lines. O₂ consumption was measured during exponential growth using an O₂ electrode. Values were normalised to the FW of aliquots taken at the same time as the O₂ measurements. Oxygen consumption by *ATCS* line 6 was not significantly different to either of the control lines (Figure 4.6). However, oxygen uptake by *ATCS* line 16 was 1.2 times that of the control lines ($p < 0.005$).

4.2.5. Analyses of citrate synthase substrates and products

Increased levels of CS could result in altered flux through the CS reaction, which could affect the steady state levels of the reaction's substrates (acetyl-CoA and oxaloacetate) and products (citrate and CoA-SH).

4.2.5.1. Acetyl-CoA.

ATCS lines 6 and 16 each had higher extractable acetyl-CoA levels than control line 6 ($p < 0.05$), but there were no significant differences between these lines and control line 4 (Figure 4.7a). Therefore, it was concluded that the over-expression of CS did not affect acetyl-CoA content. Acetyl-CoA levels were further investigated by comparing extractable acetyl-CoA with CS levels in protein extracts from the same cultures (Figure 4.7b). Linear regression indicated that the data was spread widely ($R^2 = 0.386$), but the slope of the linear regression line was significantly positive ($p < 0.001$) as calculated by Spearman correlation. This suggests that CS levels were weakly correlated with acetyl-CoA content.

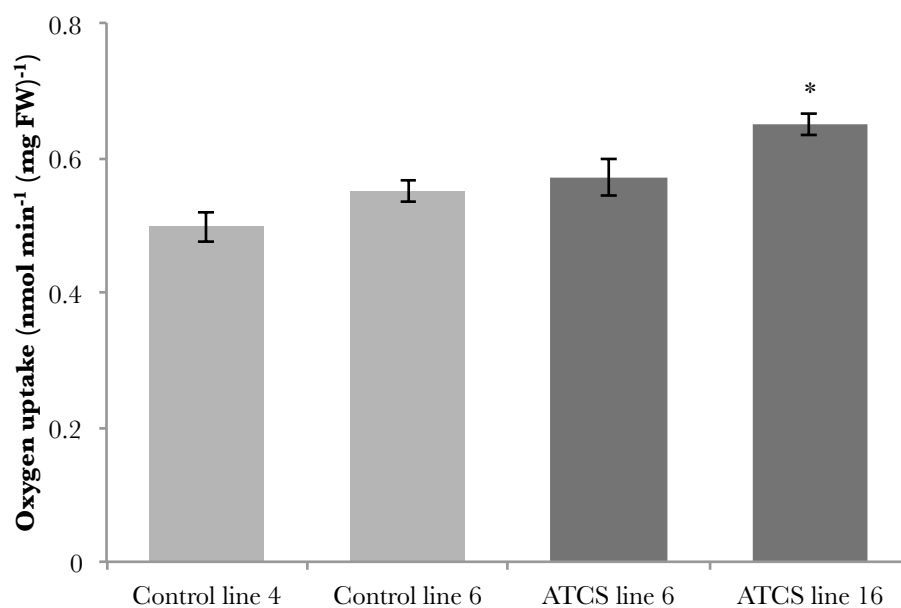


Figure 4.6. O₂ uptake by transgenic *ATCS* and control line BY-2 cell cultures during logarithmic growth.

O₂ uptake of 0.5 ml aliquots of suspension culture was recorded. FW of the corresponding suspension cultures was then measured, allowing normalisation of oxygen uptake to FW. Error bars = SEM, n = 5. “*” indicates statistically significant differences to each of the control lines (p < 0.005, student’s *t* test).

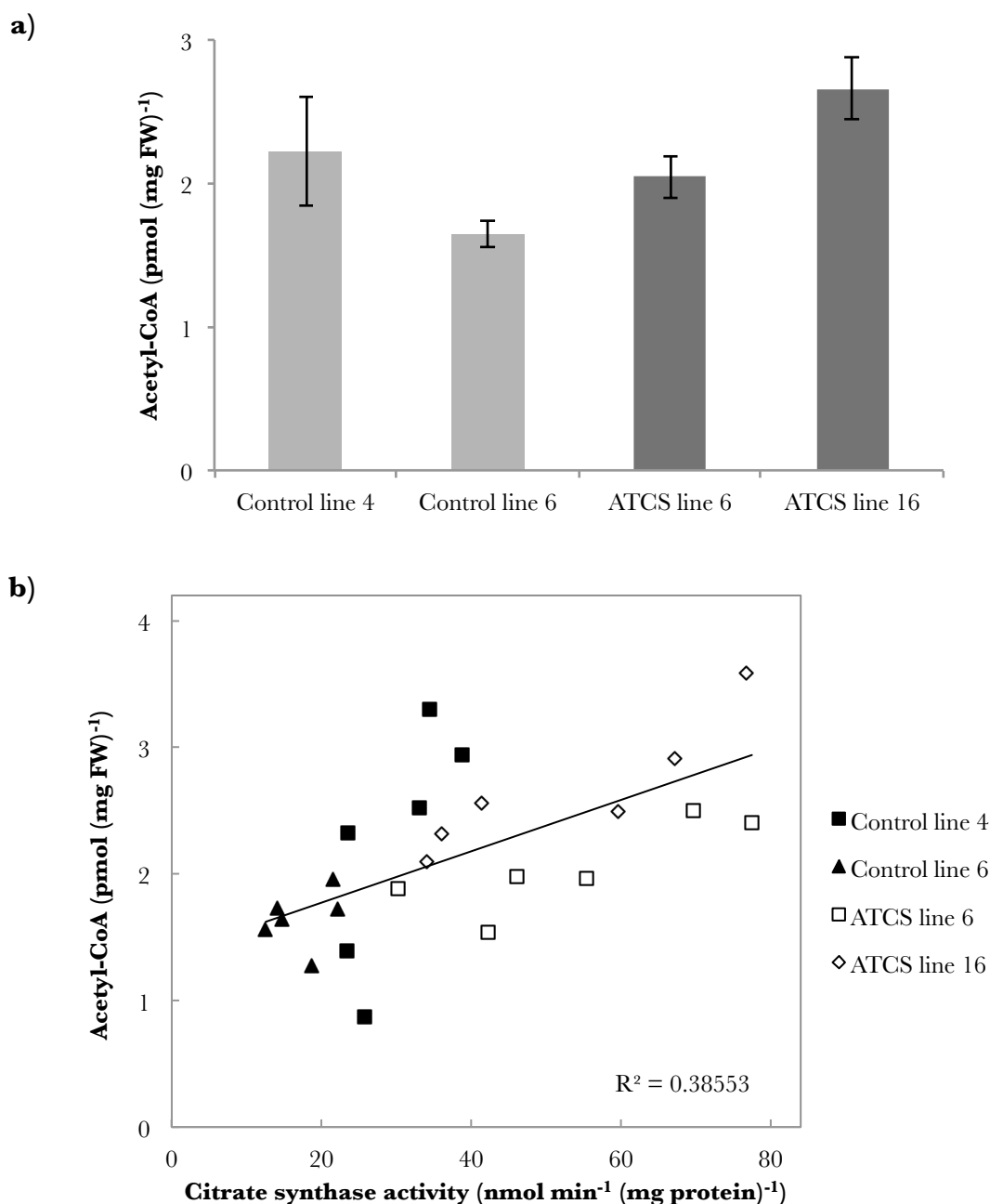


Figure 4.7. Acetyl-CoA content of transgenic *ATCS* BY-2 cell lines.

a) Acetyl-CoA content was measured in trichloroacetic acid extracts from logarithmic phase BY-2 cultures. Error bars represent SEM, $n = 6$. **b)** Acetyl-CoA content was plotted against CS activity measured in the same samples. Data points represent individual biological replicates. Acetyl-CoA measurements are the mean of three assay replicates. The linear regression formula is $y = 0.0203x + 1.37$. Spearman correlation coefficient was 0.65 indicating a significant moderate correlation ($p < 0.001$).

4.2.5.2. Citrate

Citrate was quantified by gas chromatography coupled to mass spectrometry (GC-MS) using norvaline as an internal standard (Appendix 4.5). Cellular citrate content was measured in methanol extracts from logarithmic-phase *ATCS* and control lines (Figure 4.8a). *ATCS* line 6 had a significantly lower extractable citrate content than control line 6 ($p < 0.01$) but was no different to control line 4. *ATCS* line 16 was not significantly different to either control line, although average citrate content in its extracts was 1.8 times that of *ATCS* line 6 ($p < 0.01$).

Citrate content of cell culture filtrate (CCF) from logarithmic phase *ATCS* and control lines was also measured to assess whether citrate efflux was affected. The results indicated no significant differences between the lines (Figure 4.8b).

4.2.6. *ATCS* and control line sterol content

Total and free sterols from *ATCS* and control lines were assayed at mid-log phase. This allowed conjugated sterols to be estimated by subtracting free sterol abundance from total sterol abundance (Figure 4.9a). There were no significant differences in either absolute levels or proportions of free and conjugated sterols between any of the lines. However, *ATCS* line 6 did have slightly increased total sterol levels relative to each of the other lines ($p < 0.05$), with mean total sterols 1.18 ± 0.04 times as abundant as the average control abundance.

The total sterol profile was probed further for changes in individual sterol levels. Comparisons of end product sterols (cholesterol, campesterol and stigmasterol) revealed highly significant increases in each of the *ATCS* lines compared to each of the control lines ($p < 0.005$, Figure

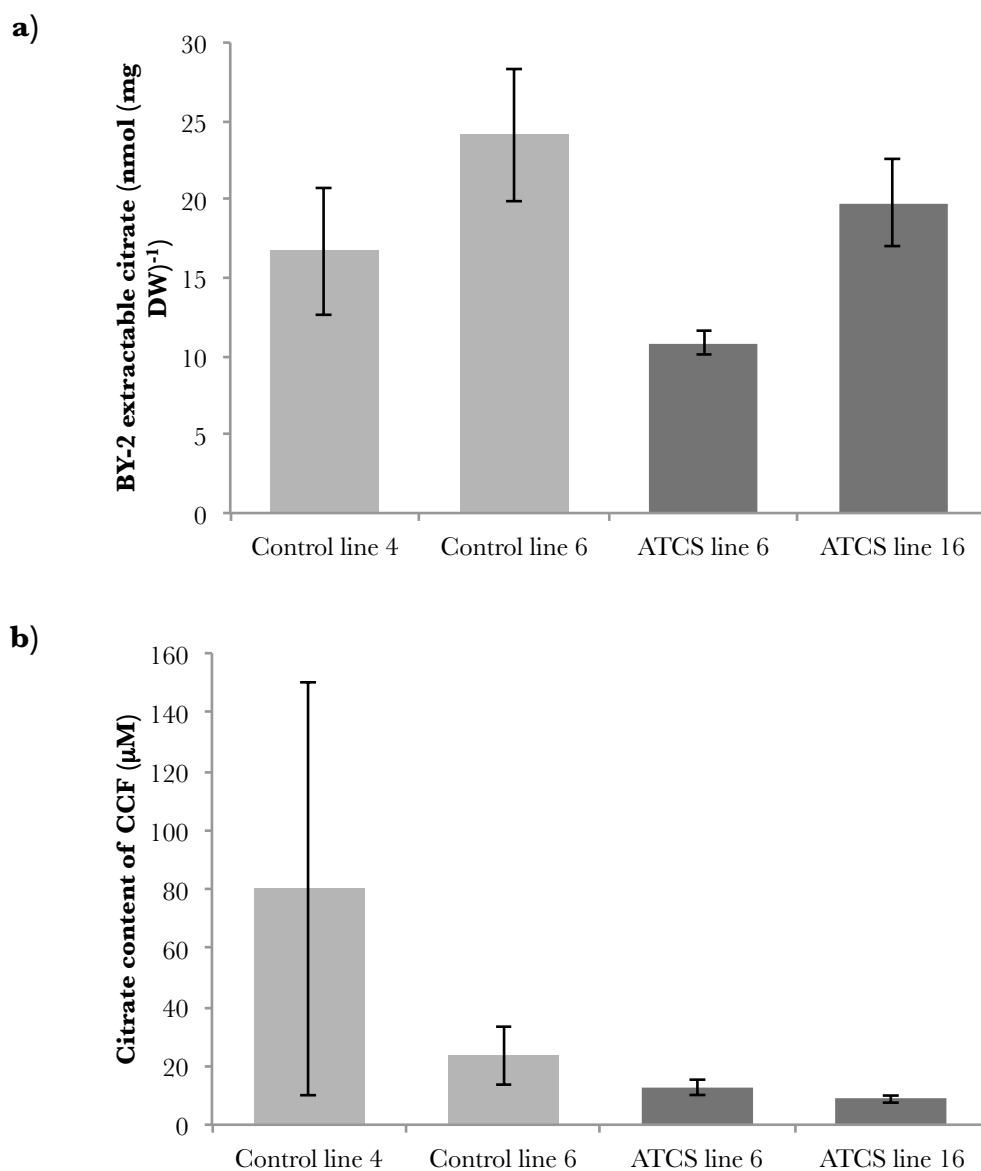


Figure 4.8. Cellular and cell culture filtrate citrate content of transgenic *ATCS* and control BY-2 cell lines.

a) Citrate content of transgenic *ATCS* BY-2 cells during logarithmic growth. $n = 11$ (control line 6, *ATCS* line 6) or 12 (control line 4, *ATCS* line 16). **b)** Citrate concentration of CCF from mid-log phase cultures following anion exchange chromatography. $n = 4$. In both graphs, error bars represent SEM.

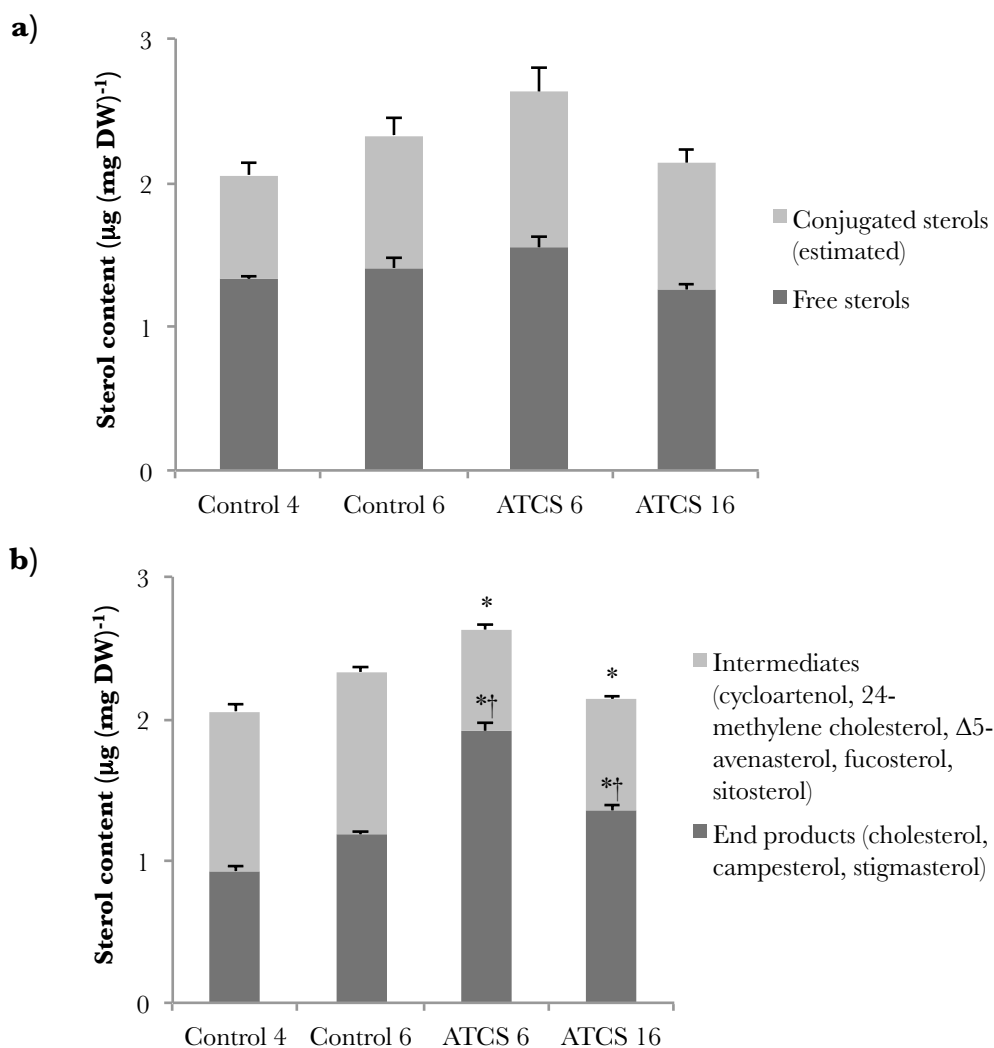


Figure 4.9. Overview of mid-log phase sterol content of transgenic *ATCS* and control BY-2 cell lines.

Total and free sterol content was measured at four days for control lines four and six, and *ATCS* line 16, and five days for *ATCS* line 6. Error bars represent SEM, $n = 3$. **a)** Free sterol and predicted conjugated sterol content. Conjugated sterols were predicted by subtracting free sterol abundance from total sterol abundance. There were no significant differences between lines when comparing free or predicted conjugated sterol levels. Total sterol measurements were significantly higher in *ATCS* line 6 than each of the other lines ($p < 0.05$, student's t test). **b)** Amounts of intermediate and pathway end product sterols were calculated from total sterol extracts. Statistically significant differences ($p < 0.05$, student's t test) between an *ATCS* line and each control line are denoted by “*”. Significant differences between *ATCS* lines 6 and 16 are denoted by “†”.

4.9b). *ATCS* line 6 end product sterol levels were 1.8 ± 0.1 times those of controls, while *ATCS* line 16 levels were 1.3 ± 0.0 times as great. *ATCS* line 6 had increases in each of these three sterols, with cholesterol, campesterol and stigmasterol levels of 5.1 ± 0.4 ($p < 0.005$), 1.6 ± 0.1 ($p < 0.005$) and 2.2 ± 0.1 ($p < 0.001$) times those of controls, respectively (Figure 4.10). *ATCS* line 16 was only significantly different in its abundance of stigmasterol ($p < 0.05$), which was increased to 2.3 times the control value. The quantifiable amounts of intermediate sterols (cycloartenol, 24-methylene cholesterol, Δ^5 -avenasterol, fucosterol and sitosterol) in *ATCS* lines 6 and 16 were reduced to 0.63 ± 0.03 and 0.70 ± 0.04 times those of control levels, respectively ($p < 0.005$). This was despite *ATCS* lines 6 and 16 having 1.7 ± 0.1 and 1.4 ± 0.1 times the control sitosterol levels, respectively ($p < 0.05$). The levels of cycloartenol, 24-methylene cholesterol, Δ^5 -avenasterol and fucosterol in *ATCS* line 6 were each significantly reduced to 0.16 ± 0.01 , 0.19 ± 0.01 , 0.59 ± 0.04 and 0.34 ± 0.01 , respectively, of the control average ($p < 0.005$ for each comparison). Cycloartenol levels were unchanged in *ATCS* line 16, but 24-methylene cholesterol, Δ^5 -avenasterol and fucosterol were reduced to 0.38 ± 0.01 , 0.36 ± 0.03 and 0.53 ± 0.01 times those of control levels, respectively ($p < 0.005$ for each comparison). These differences were also observed in measurements of free sterols, except in the case of Δ^5 -avenasterol, for which there was no significant difference between the lines (Appendix 4.6).

Comparisons of individual free sterol levels followed a similar trend to the total sterol levels (Appendix 4.6), with the consequence that this was also reflected in the estimated individual conjugated sterol levels (Appendix 4.7), and the proportions of conjugated end-of-pathway and intermediate sterols. The differences in individual sterol content had the effect of significantly increasing the proportions of campesterol, stigmasterol and sitosterol in each of the *ATCS* lines ($p < 0.005$, each), at the expense of 24-methylene cholesterol, Δ^5 -avenasterol and fucosterol ($p < 0.0005$).

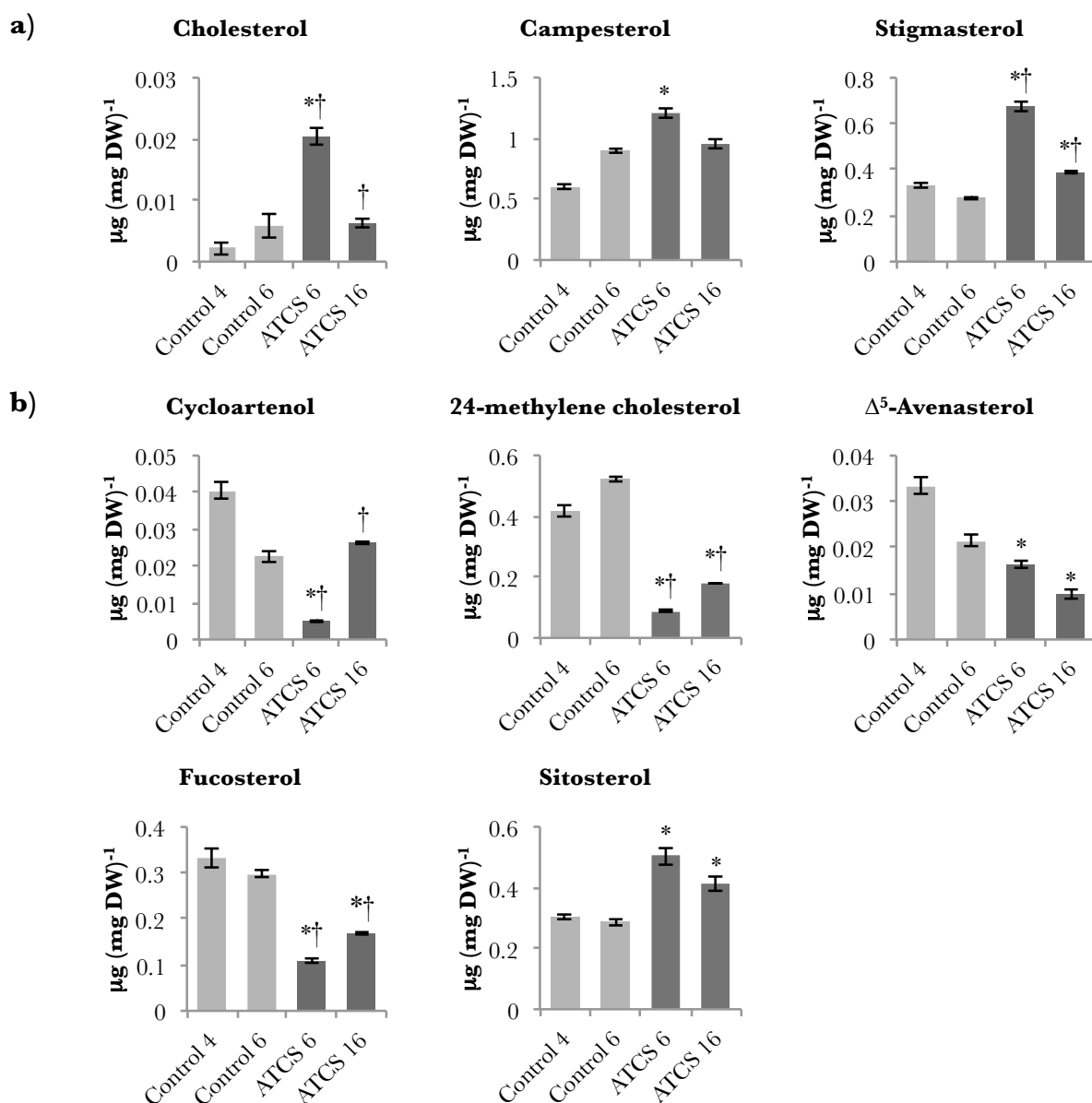


Figure 4.10. Sterol content in transgenic *ATCS* and control BY-2 cell lines.

Amounts of **a)** end-product sterols, and **b)** pathway intermediate sterols. The order is based on proximity to cycloartenol in the sterol biosynthesis pathway. Error bars represent SEM, $n = 3$. *ATCS* measurements that are statistically different to each of the control lines ($p < 0.05$, student's t test) are denoted by “*”. Significant differences between *ATCS* lines 6 and 16 are marked with “†” ($p < 0.00625$, significant following student's t test with Bonferroni-correction for multiple comparisons).

There were also significant differences in the levels of individual and total sterols between *ATCS* lines 6 and *ATCS* line 16. *ATCS* line 6 had an average total sterol content 1.2 ± 0.0 times that of *ATCS* line 16 ($p < 0.05$). This was primarily due to a 1.7 ± 0.1 times increase in stigmasterol content in *ATCS* line 6 ($p < 0.0005$). Cholesterol levels were also higher in *ATCS* line 6 ($p < 0.001$), but cycloartenol, 24-methylene cholesterol and fucosterol levels were reduced ($p < 0.0005$ each). As a result, end product sterols (stigmasterol, campesterol and cholesterol) made up 0.73 ± 0.01 of the total sterol content of *ATCS* line 6, compared to 0.63 ± 0.00 in *ATCS* line 16, and 0.45 ± 0.00 and 0.51 ± 0.00 in control lines 4 and 6, respectively (mean $\pm$ se, $n = 3$. Each line was significantly different to every other line, $p < 0.0005$.)

4.2.7. Further analyses of soluble metabolites by GC-MS

A metabolomics approach based on GC-MS metabolite profiling was used to compare methanol-soluble metabolites of the two *ATCS* lines with the two control lines. Methanol extraction was performed on four biological replicates harvested at daily intervals during mid-log to early stationary growth phases for each line (days four, five and six for control line 6 and *ATCS* line 16, and days four, five, six and seven for control line 4 and *ATCS* line 6). Metabolite extraction was performed in four batches with one replicate of each age for each line in each extraction batch. Samples from the first replicate batch typically had analyte counts of less than 50% of later replicates, and ribitol was not detected in all these samples. Because of this these samples were omitted from further analyses.

4.2.7.1. Establishment of metabolic steady state

Initial experiments were performed to determine metabolic steady state, when the levels of intermediary metabolites are unchanging. This was done to ensure that cultures were in a similar metabolic state when comparisons were made between transgenic lines. Doing this limits any effects that may be due to the growth phase of cultures rather than being due to phenotypic differences. This was considered necessary due to the slower growth rate of *ATCS* line 6. There were no differences in detected ribitol levels between age groups within each transgenic line. Detected analyte levels were normalised to ribitol as a result.

Control line 4

103 analytes were identified in at least two samples of control line 4 (Appendix 4.8). Comparing individual analyte levels between ages using paired *t* tests did not reveal any significant differences between ages after Bonferroni-correction for multiple comparisons. The data was further analysed using principal component analysis (PCA). This identified three statistically significant principal components, which together accounted for 77.2% of the variation between samples. Multivariate analysis of variance (MANOVA) of these components indicated differences between groupings ($p < 0.05$). Further pairwise MANOVA between ages indicated differences between component scores of days five and six, and days five and seven ($p < 0.05$ each). Analysis of variance (ANOVA) by individual component revealed the differences to be statistically significant only in the case of component one ($p < 0.05$), which explains 35.9% of inter-sample variance. This gave two clusters based on age: days four and five had negative scores, while days six and seven had positive scores (Figure 4.11a). Student's *t* test of component one scores for these two clusters revealed the differences to be highly significant ($p < 0.0005$).

Control line 6

98 analytes were identified in at least two samples of control line 6 (Appendix 4.9). Three principal components were identified explaining 83.3% of the variance between samples. MANOVA of these principal component scores revealed significant differences between days five and six ($p < 0.01$), and days four and six ($p < 0.005$). ANOVA of principal components revealed differences between ages for component one ($p < 0.0001$), which accounts for 39.1% of variance between samples, with six-day-old samples having the most negative scores and four-day-old samples having the most positive (Figure 4.11b). Student's t tests showed significant differences between days four and six ($p < 0.01$), and days five and six ($p < 0.05$). Days four and five were not significantly different.

ATCS line 6

97 analytes were identified in at least two samples from ATCS line 6 (Appendix 4.10). Only one of these was found to have changed significantly over the time-course of the experiment after Bonferroni-correction for multiple comparisons ($p < 0.0005$). This was glycerol-2-phosphate (4TMS), which increased by approximately five times between days five and seven. Three significant principal components were revealed by PCA, together accounting for 76.3% of inter-sample variance. MANOVA of these principal components revealed differences between days four and six ($p < 0.01$; Figure 4.11c). Individual differences between ages were revealed by MANOVA in principal component three, which accounts for 17.2% of inter-sample variance ($p < 0.05$). Student's t tests showed that the principal component three scores of day seven were different to each other day ($p < 0.05$). Additional t tests exposed differences between days four and five in principal component two, which accounted for 23.0% of inter-sample variance ($p < 0.05$). No statistical analyses revealed significant differences between days five and six.

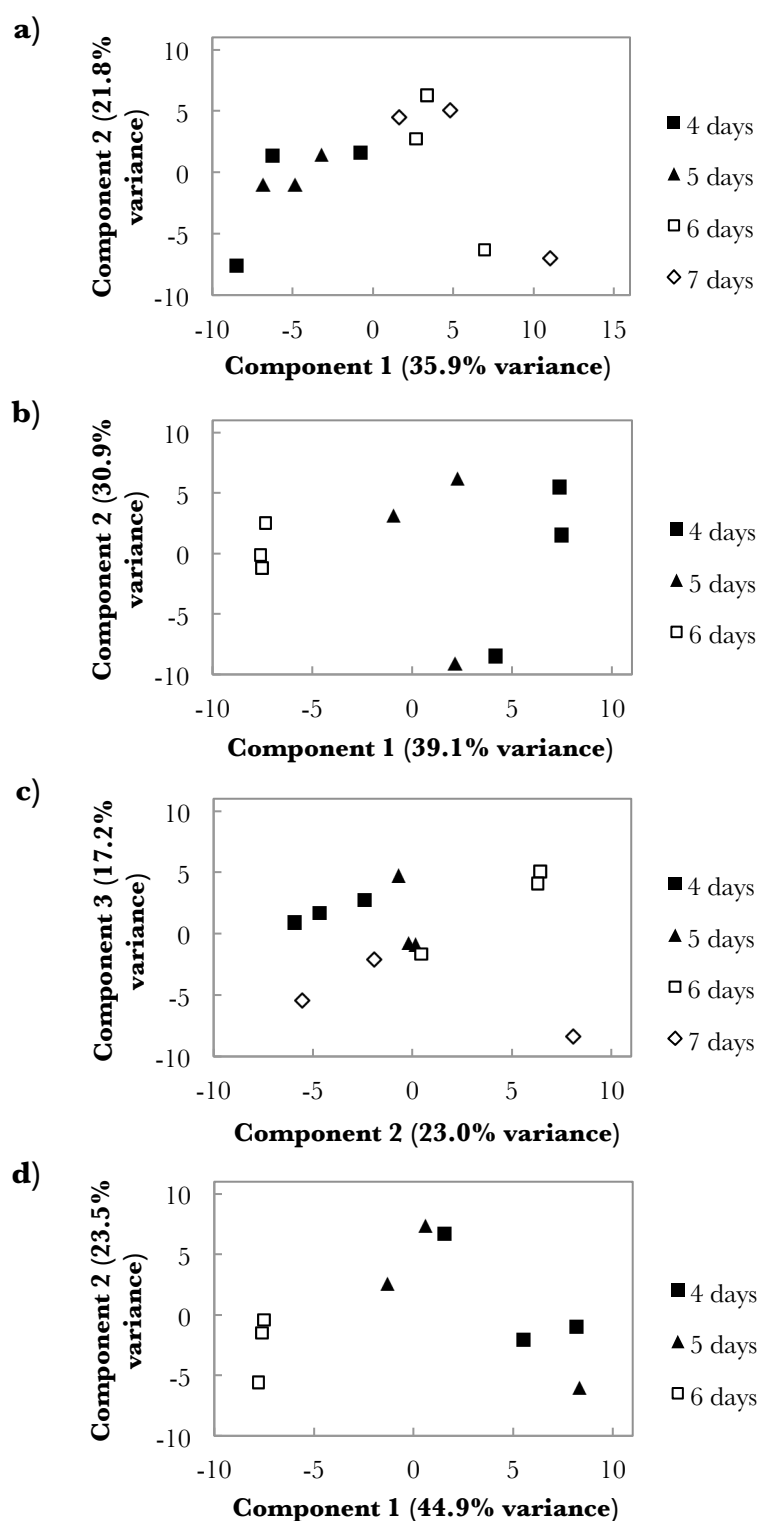


Figure 4.11. Principal component analysis scores plots of methanol-soluble metabolite data for transgenic control and *ATCS* BY-2 cell lines by age.

Methanol-soluble metabolites were identified using a TagFinder-based GC-MS approach and were quantified by normalisation to the internal standard, ribitol, and pre-extraction sample dry weight (DW). **a)** Control line 4. Principal component three, explaining 19.6% of inter-sample variance, not shown. **b)** Control line 6. Principal component three (13.2% variance) not shown. **c)** *ATCS* line 6. Principal component one (36.2% variance) not shown. **d)** *ATCS* line 16.

***ATCS* line 16**

96 analytes were identified in at least two samples from *ATCS* line 16 (Appendix 4.11). None of these altered significantly over the time-course of the experiment when using Bonferroni-correction for multiple replicates. PCA identified two significant principal components explaining 68.4% of variance (Figure 4.11d). Differences between four and six-day-old cultures ($p < 0.01$), and five and six-day-old cultures ($p < 0.05$) were discovered by MANOVA of these principal components. ANOVA revealed significant differences in principal component one ($p < 0.01$). Days five and six differed in this component ($p < 0.05$). Days four and five did not differ in any measurement.

4.2.7.2. Comparison of aging between transgenic lines

Analyte loadings for the principal component most explanatory of age were investigated to get an overview of the age-associated changes in metabolite profile in each transgenic line. These were components one for the two control lines and *ATCS* line 16, and component three for *ATCS* line 6. Loadings varied between -0.17 and 0.19. -0.8 and 0.8 were chosen as arbitrary cut-offs for determining if analytes were strongly positively correlated with age, relatively unchanged or strongly negatively correlated with age (Table 4.1). Analytes were classed as sugars, amino acids, organic acids, sterols, triacylglycerol components or other metabolites. The proportions of each metabolite class that were positively, not, or negatively correlated with age in each line were plotted (Figure 4.12). Amino acids were largely positively correlated with age in the two control lines, whereas 50% were unchanged in the two *ATCS* lines, and only 25% and 38% were correlated with age in *ATCS* lines 6 and 16, respectively. Most sugars were uncorrelated with age, except in *ATCS* line 16, in which case 17 of the 20 sugars were negatively correlated with age. Triacylglycerol components were primarily unaffected by age in the two control lines, but were mainly negatively correlated with age in the two *ATCS* lines.

Table 4.1. Numbers of analytes that change with age in transgenic *ATCS* and control *BY-2* cell lines.

Analyte loadings for the principal component most explanatory of differences between age groups in each line were investigated. Except in the case of control line 4, analytes with loadings of less than -0.8 were considered to be positively correlated with age, analytes with loadings between -0.8 and 0.8 were considered to be unaffected by age, and analytes with loadings greater than 0.8 were considered to be negatively correlated with age. Control line 4 loadings were the inverse, as age was positively correlated with principal component one.

	Control 4	Control 6	<i>ATCS</i> 6	<i>ATCS</i> 16
Positively correlated with age	45	41	17	17
Relatively unchanged	47	37	46	31
Negatively correlated with age	11	20	34	48
Total	103	98	97	96

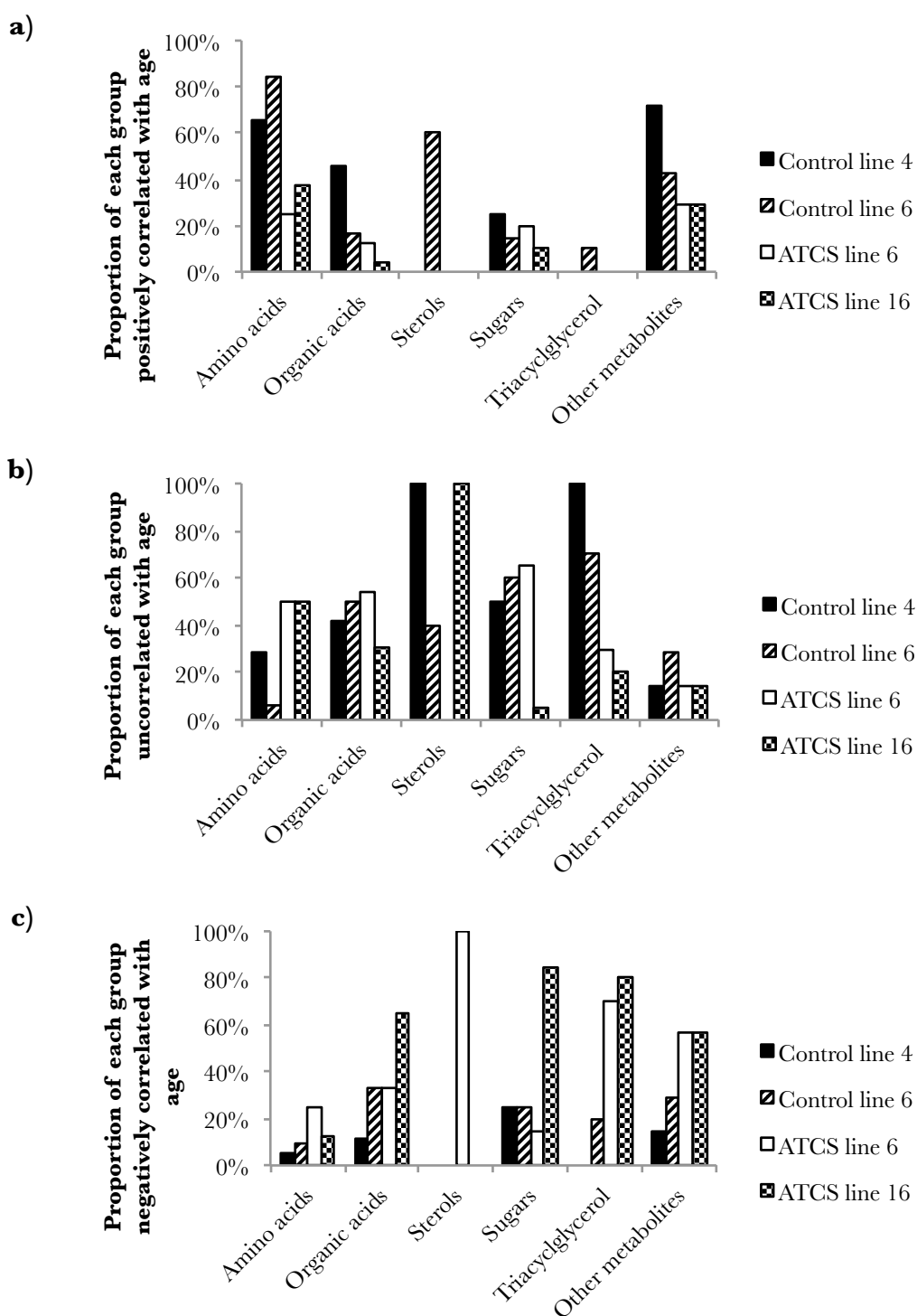


Figure 4.12. Age-related metabolite changes in transgenic control and *ATCS* BY-2 cell lines.

Analytes were categorised by their loadings for principal components explaining harvesting age-related variation for each transgenic cell line. Analytes were then classified as belonging to a particular metabolite group. The proportions of each metabolite group either **a)** positively, **b)** not, or **c)** negatively correlated with age were calculated.

About half of the organic acids in each line were uncorrelated with age. However, 15 of the 23 were negatively correlated with age in *ATCS* line 16 samples, and 12 of 26 were positively correlated in control line 4. Slightly more were negatively correlated with age than positively correlated with age in control line 6 and *ATCS* line 6.

Aging was further compared between lines by calculating sum of squared differences between loadings for each analyte of each line. The two control lines were the most similar to each other, with a score of 0.56. *ATCS* line 16 was more similar to control line 6 than four, with scores of 0.95 and 1.56, respectively. *ATCS* line 6 was most dissimilar to each of the control lines, with scores of 2.20 and 2.17 against control lines 4 and 6, respectively. It was more similar to *ATCS* line 16, with a score of 1.56.

4.2.7.3. Comparisons of metabolite levels during logarithmic growth

Following establishment of metabolic steady state, methanol extracts from two statistically indistinguishable time-points from the logarithmic growth phase were compared between lines. These were days four and five for the two control lines and *ATCS* line 16, and days five and six for *ATCS* line 6. Analytes identified in fewer than two samples were ignored. This led to the analysis of 103 chemical derivatives comprising 82 metabolites. Initial analyses revealed that ribitol content of *ATCS* line 16 extracts was $49\% \pm 6\%$ of the average control value ($p < 0.05$), with the effect that the sum of peak areas normalised to ribitol and DW in *ATCS* line 16 extracts was 2.4 ± 0.4 times that of control extracts ($p < 0.05$ following student's *t* test comparisons with each control line). By contrast, the peak areas of the alkanes (added during derivatisation to allow normalisation of retention time between samples) and the sum of analyte peak areas were no different to other lines, at $106\% \pm 7\%$ and $107\% \pm 9\%$,

respectively. In order for the results to truly represent a doubling in methanol-soluble metabolite content of *ATCS* line 16, the recovery from this line must have been roughly half of the recovery from the other lines. Analyses of metabolome data normalised to ribitol and DW are presented in Appendix 4.12. However, it seems unlikely for recovery to be directly inversely proportional to the degree of metabolite increase.

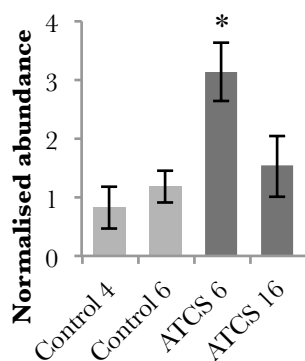
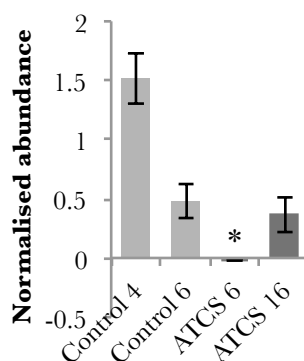
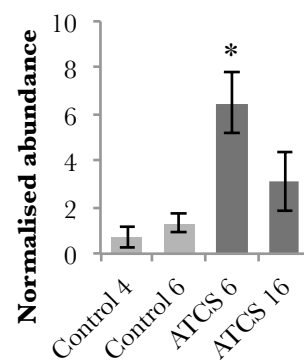
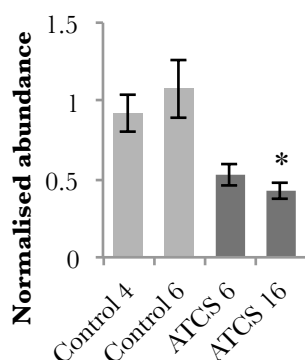
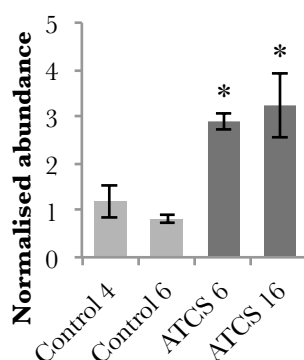
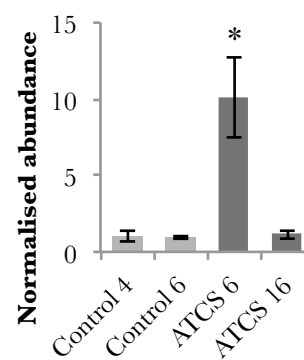
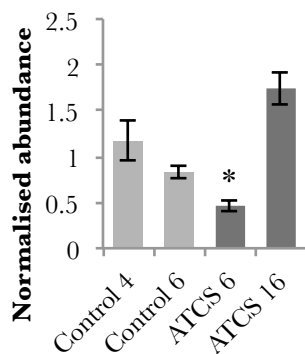
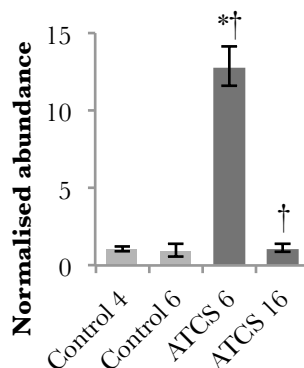
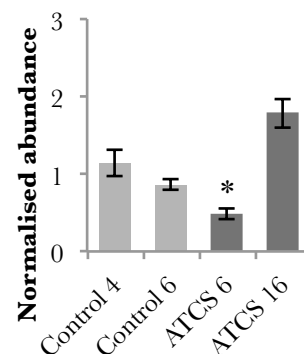
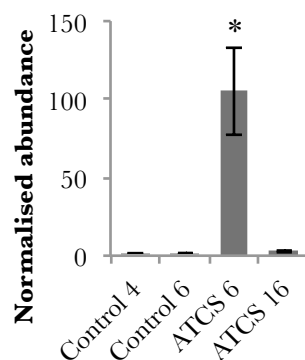
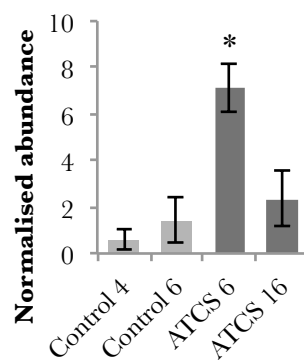
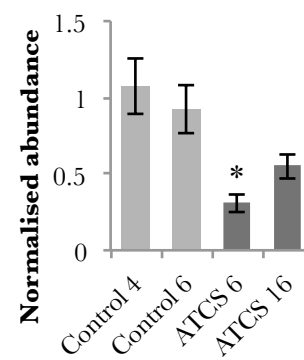
As a second means of analysing metabolite abundance between lines, peak areas were normalised to the sum of the alkane peak areas in each sample. This allowed normalisation to GC-MS detector response and DW, while ignoring the potentially unreliable normalisation to recovery. Tetradecane was not found in all samples, so this was ignored from alkane sum calculations. Alkanes included were dodecane, pentadecane, docosane, octacosane, dotriacontane and hexatriacontane, which were each identified in all samples. The same approach was used to reanalyse the metabolome differences between ages for each of the transgenic lines, but this did not give significantly different results to those previously discussed.

As a means of limiting the likelihood of false negatives resulting from Bonferroni correction for multiple comparisons, analytes were judged to have altered levels in *ATCS* lines if each of the p-values arising from student's *t* tests between the test line and each of the control lines were less than 0.05, and if the product of these p-values was less than the critical p-value after Bonferroni correction (approximately 0.0005). This revealed that 10 analytes (asparagine [-H₂O] (2TMS), ethanolamine (3TMS), galactinol (9TMS), α -1-O-methyl-galactopyranoside (4TMS), D-glucopyranose (5TMS), glucose-6-phosphate (6TMS), DL-glutamine (4TMS), serine (2TMS), serine (4TMS) and DL-threonine (2TMS)) were significantly more abundant in *ATCS* line 6 extracts than control extracts, while six (aspartic acid (3TMS), the second elution of galactose-6-phosphate (1MEOX) (6TMS), glucose-6-phosphate (1MEOX) (6TMS) MP, glycerol-3-phosphate (4TMS), *myo*-inositol-1-phosphate (7TMS) and xylulose-5-phosphate

(MEOX) (5TMS) BP) were less abundant (Figure 4.13; Table 4.2). Two analytes (galactinol (9TMS) and uracil (2TMS)) were more abundant in *ATCS* line 16 samples than controls, and one (fumaric acid (2TMS)) was less abundant. Therefore, galactinol (9TMS) was the only analyte consistently found at altered levels in *ATCS* extracts compared to the control extracts.

The metabolome data was probed further to determine how the altered levels of different TMS and MEOX derivatives reflect changes in metabolite levels in the *ATCS* lines. Ethanolamine, galactinol, glycerol-3-phosphate, *myo*-inositol-1-phosphate and xylulose-5-phosphate each had only one derivative. Therefore changes in the abundance of their derivatives in *ATCS* line 6 were accepted to reflect altered *in vivo* concentrations. Similarly, only one derivative each of fumarate and uracil was detected, so the respective decrease and increase in these derivatives in *ATCS* line 16 extracts was accepted to reflect altered *in vivo* concentrations.

α -1-O-methylgalactopyranoside also had only one derivative. However, this compound is not found in literature on plant metabolites. 1,2- α -L-fucosidase from almond (*Prunus dulcis*) hydrolyses methyl-2- α -L-fucopyranosyl- β -D-galactopyranoside into 6-deoxy-L-galactose and methyl- β -D-galactopyranoside (Ogata-Arakawa et al., 1977). However, methyl- β -D-galactopyranoside is a stereoisomer of α -1-O-methyl-galactopyranoside with a distinct mass spectrum of the 4TMS derivative. It is possible that methyl-galactopyranoside was formed during methanol extraction by methylation of galactopyranose (Sasaki et al., 2005). Such a method would be expected to methylate galactopyranose in the β orientation approximately as often as in the α orientation. β -1-O-methyl-galactopyranoside (4TMS) was initially identified during the peak assignment process in TagFinder, but this was omitted from further analyses due to the match being considered poor. The putative β -1-O-methyl-galactopyranoside peaks were $22\% \pm 2\%$ (mean $\pm$ SEM, $n = 20$) of the size of

Asparagine [-H₂O] (2TMS)**Aspartate (3TMS)****Ethanolamine (3TMS)****Fumarate (2TMS)****Galactinol (9TMS)****Galactopyranoside, 1-O-methyl-, alpha- (4TMS)****Galactose-6-phosphate (1MEOX) (6TMS) late peak****Glucopyranose, D- (5TMS)****Glucose-6-phosphate (1MEOX) (6TMS) MP****Glucose-6-phosphate (6TMS)****Glutamine, DL- (4TMS)****Glycerol-3-phosphate (4TMS)**

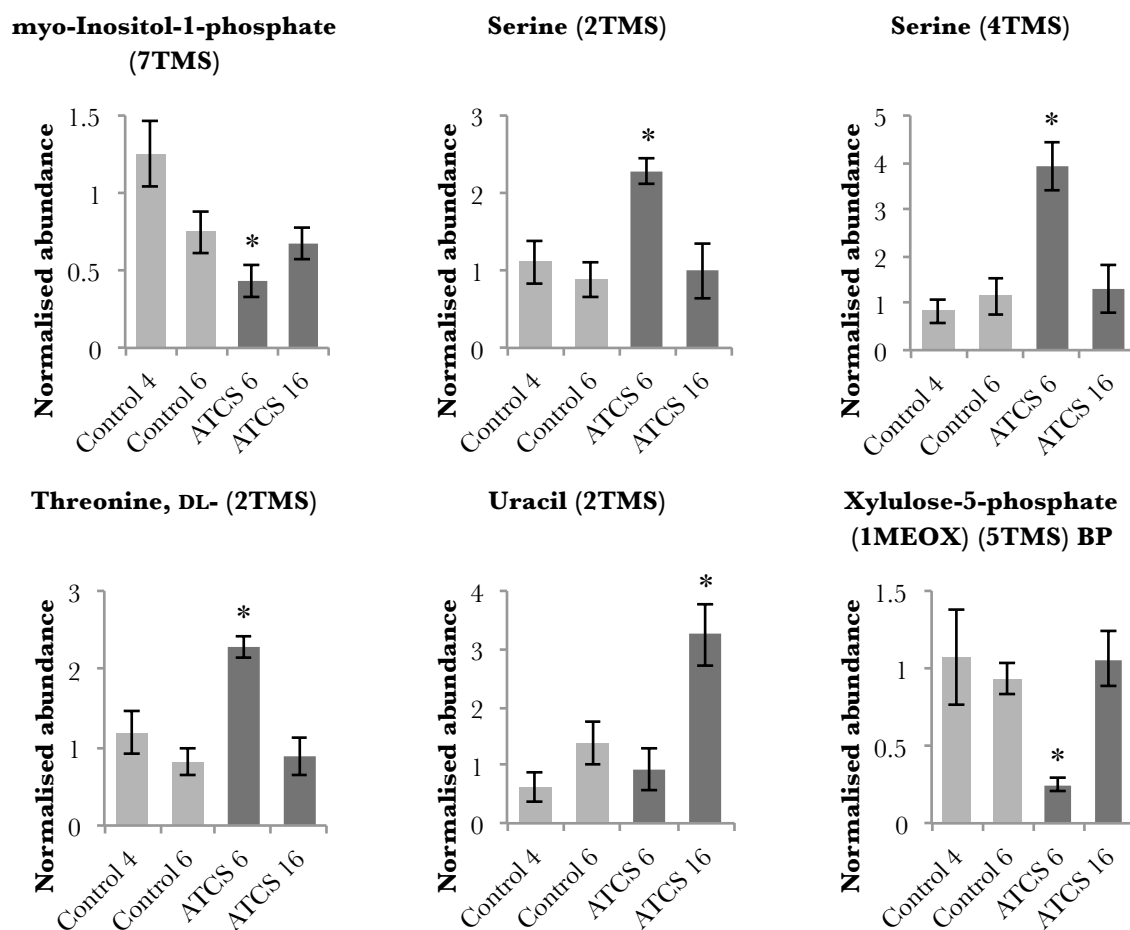


Figure 4.13. Analytes at altered levels in methanol extracts from *ATCS* BY-2 cell line 6 or 16 (and previous page).

TMS and MEOX derivatives of metabolites at significantly different levels in *ATCS* line 6 or 16 extracts than extracts from each of the control lines are marked with “*” (product of p-values in student’s *t* tests between *ATCS* line 6 and each of the two control lines < 0.0005; p-value of each test between *ATCS* line 6 and each of the two control lines < 0.05). Bars marked with “†” denote significant differences between the two *ATCS* lines (p < 0.0005). (0.0005 is critical p-value after Bonferroni-correction for multiple comparisons. Values were normalised to the control mean value. Error bars represent SEM, n = 6.)

Table 4.2. Metabolome comparisons between transgenic *ATCS* and control BY-2 cell lines.

Values derived by normalising TagFinder-derived GC-MS intensity values to the sum of the abundance of alkane standards added during derivatisation, followed by normalisation to DW. Values for each analyte were then normalised to the average control value for that analyte. Normalised abundances were compared between transgenic lines using student's *t* tests. Values in bold indicate significant differences to each control line ($p < 0.05$ compared to each control line; product of *p*-values compared to each control line < 0.0005 , the critical *p*-value after Bonferroni correction). Underlined values indicate significant differences between the two *ATCS* lines ($p < 0.0005$).

Analyte	Normalised abundance (mean $\pm$ SEM)			
	Control 4	Control 6	<i>ATCS</i> 6	<i>ATCS</i> 16
Aconitate, cis- (3TMS)	0.87 $\pm$ 0.29	1.13 $\pm$ 0.10	0.34 $\pm$ 0.23	1.02 $\pm$ 0.46
Alanine (2TMS)	1.88 $\pm$ 1.88	0.12 $\pm$ 0.12	0.00 $\pm$ 0.00	0.40 $\pm$ 0.40
Alanine (3TMS)	1.02 $\pm$ 0.36	0.98 $\pm$ 0.13	1.76 $\pm$ 0.35	1.09 $\pm$ 0.18
Alanine [+CO ₂] (2TMS)	0.80 $\pm$ 0.28	1.20 $\pm$ 0.18	1.22 $\pm$ 0.35	1.48 $\pm$ 0.21
Alanine, β - (3TMS)	1.20 $\pm$ 0.39	0.80 $\pm$ 0.05	1.13 $\pm$ 0.07	1.27 $\pm$ 0.23
Arabinose (1MEOX) (4TMS) MP	1.14 $\pm$ 0.28	0.86 $\pm$ 0.10	0.50 $\pm$ 0.05	0.88 $\pm$ 0.14
Asparagine (2TMS)	1.13 $\pm$ 0.28	0.87 $\pm$ 0.10	1.61 $\pm$ 0.29	0.87 $\pm$ 0.28
Asparagine (3TMS)	1.43 $\pm$ 0.56	0.57 $\pm$ 0.15	1.00 $\pm$ 0.32	1.06 $\pm$ 0.36
Asparagine [-H ₂ O] (2TMS)	0.82 $\pm$ 0.35	1.18 $\pm$ 0.27	3.13 $\pm$ 0.49	1.53 $\pm$ 0.52
Aspartate (2TMS)	1.16 $\pm$ 0.95	0.84 $\pm$ 0.38	0.94 $\pm$ 0.23	0.33 $\pm$ 0.32
Aspartate (3TMS)	1.52 $\pm$ 0.22	0.48 $\pm$ 0.14	-0.02 $\pm$ 0.01	0.37 $\pm$ 0.15
Butanoate, 2-amino- (2TMS)	1.85 $\pm$ 1.85	0.15 $\pm$ 0.15	0.00 $\pm$ 0.00	0.47 $\pm$ 0.47
Butanoate, 2,4-dihydroxy- (3TMS)	1.24 $\pm$ 0.31	0.76 $\pm$ 0.09	0.58 $\pm$ 0.08	0.62 $\pm$ 0.09
Butanoate, 4-amino- (3TMS)	0.80 $\pm$ 0.18	1.20 $\pm$ 0.47	0.40 $\pm$ 0.05	1.21 $\pm$ 0.34
Butanoic acid, 4-hydroxy- (2TMS)	0.01 $\pm$ 0.05	1.99 $\pm$ 1.25	-0.06 $\pm$ 0.03	3.43 $\pm$ 1.89
Butyro-1,4-lactam (1TMS)	0.20 $\pm$ 0.26	1.80 $\pm$ 1.05	0.27 $\pm$ 0.04	1.76 $\pm$ 0.52
Campesterol (1TMS)	0.91 $\pm$ 0.41	1.09 $\pm$ 0.47	1.34 $\pm$ 0.51	1.44 $\pm$ 0.54
Citric acid (4TMS)	0.88 $\pm$ 0.33	1.12 $\pm$ 0.25	0.81 $\pm$ 0.11	1.45 $\pm$ 0.45
Cycloartenol (1TMS)	1.22 $\pm$ 0.89	0.78 $\pm$ 0.78	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Dehydroascorbic acid dimer (2MEOX) MP	1.44 $\pm$ 0.50	0.56 $\pm$ 0.07	0.18 $\pm$ 0.03	0.50 $\pm$ 0.07
Eicosanoic acid (1TMS)	-4.21 $\pm$ 15.1	6.21 $\pm$ 19.6	0.78 $\pm$ 5.27	-5.23 $\pm$ 10.7
Erythronic acid (4TMS)	1.27 $\pm$ 0.36	0.73 $\pm$ 0.09	0.47 $\pm$ 0.03	0.68 $\pm$ 0.08
Ethanolamine (3TMS)	0.71 $\pm$ 0.44	1.29 $\pm$ 0.41	6.49 $\pm$ 1.29	3.08 $\pm$ 1.29
Fructose (1MEOX) (5TMS) BP	1.64 $\pm$ 1.30	0.36 $\pm$ 0.05	0.21 $\pm$ 0.04	0.48 $\pm$ 0.11
Fructose (1MEOX) (5TMS) MP	1.75 $\pm$ 1.56	0.25 $\pm$ 0.08	0.02 $\pm$ 0.04	0.29 $\pm$ 0.13
Fucosterol (1TMS)	1.02 $\pm$ 0.47	0.98 $\pm$ 0.47	0.18 $\pm$ 0.13	0.34 $\pm$ 0.17
Fumaric acid (2TMS)	0.92 $\pm$ 0.12	1.08 $\pm$ 0.19	0.53 $\pm$ 0.07	0.42 $\pm$ 0.06
Galactinol (9TMS)	1.19 $\pm$ 0.36	0.81 $\pm$ 0.09	2.88 $\pm$ 0.16	3.24 $\pm$ 0.68
Galactopyranoside, 1-O-methyl-, α - (4TMS)	1.03 $\pm$ 0.39	0.97 $\pm$ 0.14	10.1 $\pm$ 2.67	1.15 $\pm$ 0.28
Galactose-6-phosphate (1MEOX) (6TMS) early peak (38.3 min)	1.42 $\pm$ 0.41	0.58 $\pm$ 0.07	0.39 $\pm$ 0.05	1.17 $\pm$ 0.21
Galactose-6-phosphate (1MEOX) (6TMS) late peak (38.9 min)	1.17 $\pm$ 0.21	0.83 $\pm$ 0.07	0.46 $\pm$ 0.07	1.74 $\pm$ 0.18
Gluconic acid (6TMS)	1.19 $\pm$ 0.25	0.81 $\pm$ 0.11	1.38 $\pm$ 0.20	0.41 $\pm$ 0.07
Glucopyranose, D- (5TMS)	1.06 $\pm$ 0.14	0.94 $\pm$ 0.37	12.8 $\pm$ 1.24	<u>1.14 $\pm$ 0.27</u>
Glucose (1MEOX) (5TMS) BP	0.94 $\pm$ 0.25	1.06 $\pm$ 0.07	0.76 $\pm$ 0.07	1.31 $\pm$ 0.20
Glucose (1MEOX) (5TMS) MP	1.63 $\pm$ 1.39	0.37 $\pm$ 0.15	-0.21 $\pm$ 0.09	0.43 $\pm$ 0.21
Glucose-6-phosphate (1MEOX) (6TMS) MP	1.14 $\pm$ 0.17	0.86 $\pm$ 0.07	0.48 $\pm$ 0.07	1.78 $\pm$ 0.19
Glucose-6-phosphate (6TMS)	0.63 $\pm$ 0.31	1.37 $\pm$ 0.28	105 $\pm$ 27.7	2.71 $\pm$ 0.54
Glutamic acid (2TMS)	1.74 $\pm$ 1.25	0.26 $\pm$ 0.07	0.06 $\pm$ 0.05	0.11 $\pm$ 0.02

Analyte	Normalised abundance (mean $\pm$ SEM)			
	Control 4	Control 6	ATCS 6	ATCS 16
Glutamic acid (3TMS)	1.35 $\pm$ 0.10	0.65 $\pm$ 0.14	0.55 $\pm$ 0.05	0.92 $\pm$ 0.20
Glutamine [-H ₂ O] (2TMS) BP	0.50 $\pm$ 0.29	1.50 $\pm$ 0.75	0.95 $\pm$ 0.29	1.34 $\pm$ 0.57
Glutamine [-H ₂ O] (3TMS) MP	0.62 $\pm$ 0.37	1.38 $\pm$ 0.54	3.54 $\pm$ 0.95	3.82 $\pm$ 1.68
Glutamine, DL- (3TMS)	0.93 $\pm$ 0.93	1.07 $\pm$ 0.70	2.50 $\pm$ 1.25	1.29 $\pm$ 1.18
Glutamine, DL- (4TMS)	0.59 $\pm$ 0.42	1.41 $\pm$ 0.99	7.08 $\pm$ 1.03	2.36 $\pm$ 1.18
Glutaric acid, 2-oxo- (1MEOX) (2TMS) MP	1.25 $\pm$ 0.25	0.75 $\pm$ 0.07	0.68 $\pm$ 0.13	1.61 $\pm$ 0.22
Glyceric acid (3TMS)	1.24 $\pm$ 0.31	0.76 $\pm$ 0.05	1.33 $\pm$ 0.09	0.77 $\pm$ 0.13
Glycerol-2-phosphate (4TMS)	0.26 $\pm$ 0.26	1.74 $\pm$ 1.02	0.14 $\pm$ 0.20	0.00 $\pm$ 0.10
Glycerol-3-phosphate (4TMS)	1.08 $\pm$ 0.18	0.92 $\pm$ 0.16	0.31 $\pm$ 0.06	0.55 $\pm$ 0.08
Glycerophosphoglycerol (5TMS)	1.20 $\pm$ 0.69	0.80 $\pm$ 0.34	0.06 $\pm$ 0.02	0.42 $\pm$ 0.22
Glycine (2TMS)	2.00 $\pm$ 2.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	2.99 $\pm$ 2.99
Glycine (3TMS)	1.00 $\pm$ 0.29	1.00 $\pm$ 0.24	2.26 $\pm$ 0.33	1.09 $\pm$ 0.31
Heptadecanoic acid (1TMS)	0.99 $\pm$ 0.55	1.01 $\pm$ 0.35	0.72 $\pm$ 0.33	0.87 $\pm$ 0.40
Hexadecanoic acid (1TMS)	0.88 $\pm$ 0.35	1.12 $\pm$ 0.47	0.39 $\pm$ 0.11	0.45 $\pm$ 0.21
Homoserine (3TMS)	0.81 $\pm$ 0.15	1.19 $\pm$ 0.30	1.47 $\pm$ 0.14	2.12 $\pm$ 0.89
Inosine (4TMS)	1.38 $\pm$ 1.38	0.62 $\pm$ 0.62	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Inositol, <i>myo</i> - (6TMS)	1.23 $\pm$ 0.27	0.77 $\pm$ 0.04	0.74 $\pm$ 0.07	0.94 $\pm$ 0.15
Isoleucine (1TMS)	0.98 $\pm$ 0.25	1.02 $\pm$ 0.14	1.39 $\pm$ 0.09	1.30 $\pm$ 0.27
Isomaltose (1MEOX) (8TMS) BP	1.51 $\pm$ 0.69	0.49 $\pm$ 0.22	0.69 $\pm$ 0.17	1.07 $\pm$ 0.24
Lactic acid, DL- (2TMS)	1.96 $\pm$ 1.96	0.04 $\pm$ 0.03	0.00 $\pm$ 0.00	0.76 $\pm$ 0.76
Leucine (1TMS)	0.89 $\pm$ 0.23	1.11 $\pm$ 0.13	1.49 $\pm$ 0.11	1.86 $\pm$ 0.39
Lyxonic acid (5TMS)	1.02 $\pm$ 0.33	0.98 $\pm$ 0.17	0.67 $\pm$ 0.12	2.09 $\pm$ 0.31
Lyxose (1MEOX) (4TMS) MP	1.02 $\pm$ 0.26	0.98 $\pm$ 0.07	0.63 $\pm$ 0.07	0.73 $\pm$ 0.11
Malic acid (3TMS)	1.25 $\pm$ 0.40	0.75 $\pm$ 0.04	0.61 $\pm$ 0.05	0.80 $\pm$ 0.16
Malic acid, 2-methyl- (3TMS)	1.28 $\pm$ 0.30	0.72 $\pm$ 0.04	0.49 $\pm$ 0.08	1.18 $\pm$ 0.20
Maltose (1MEOX) (8TMS) BP	0.89 $\pm$ 0.24	1.11 $\pm$ 0.21	1.25 $\pm$ 0.08	2.70 $\pm$ 0.73
Maltose (1MEOX) (8TMS) MP	0.27 $\pm$ 0.07	1.73 $\pm$ 0.80	0.39 $\pm$ 0.02	5.03 $\pm$ 1.98
Methionine (1TMS)	1.04 $\pm$ 0.39	0.96 $\pm$ 0.33	3.24 $\pm$ 0.85	1.41 $\pm$ 0.61
Methionine (2TMS)	0.94 $\pm$ 0.14	1.06 $\pm$ 0.20	2.54 $\pm$ 0.61	3.49 $\pm$ 1.10
<i>myo</i> -Inositol-1-phosphate (7TMS)	1.25 $\pm$ 0.21	0.75 $\pm$ 0.14	0.43 $\pm$ 0.11	0.68 $\pm$ 0.10
Norleucine (2TMS)	1.55 $\pm$ 1.09	0.45 $\pm$ 0.08	0.44 $\pm$ 0.05	0.59 $\pm$ 0.10
Octadecadienoic acid, n- (1TMS)	0.73 $\pm$ 0.35	1.27 $\pm$ 0.69	0.61 $\pm$ 0.18	0.43 $\pm$ 0.16
Octadecan-1-ol, n- (1TMS)	0.52 $\pm$ 1.03	1.48 $\pm$ 1.26	0.04 $\pm$ 0.54	-0.66 $\pm$ 0.58
Octadecanoic acid (1TMS)	1.14 $\pm$ 0.60	0.86 $\pm$ 0.18	0.69 $\pm$ 0.14	0.70 $\pm$ 0.21
Ornithine-1,5-lactam (2TMS)	1.67 $\pm$ 0.84	0.33 $\pm$ 0.16	0.16 $\pm$ 0.16	0.29 $\pm$ 0.21
Phenylalanine (2TMS)	0.79 $\pm$ 0.16	1.21 $\pm$ 0.17	0.62 $\pm$ 0.08	0.65 $\pm$ 0.18
Phosphoric acid (3TMS)	1.43 $\pm$ 0.79	0.57 $\pm$ 0.09	0.15 $\pm$ 0.03	0.53 $\pm$ 0.15
Putrescine (4TMS)	1.14 $\pm$ 0.32	0.86 $\pm$ 0.22	1.98 $\pm$ 0.20	1.39 $\pm$ 0.53
Pyroglutamic acid (2TMS)	1.08 $\pm$ 0.25	0.92 $\pm$ 0.15	0.85 $\pm$ 0.10	1.15 $\pm$ 0.30
Ribonic acid (5TMS) early peak (29.1 min)	1.15 $\pm$ 0.34	0.85 $\pm$ 0.06	0.52 $\pm$ 0.03	0.47 $\pm$ 0.07
Ribonic acid (5TMS) late peak (29.2 min)	1.06 $\pm$ 0.20	0.94 $\pm$ 0.12	0.48 $\pm$ 0.03	0.81 $\pm$ 0.09
Ribose (1MEOX) (4TMS) BP	1.06 $\pm$ 0.25	0.94 $\pm$ 0.07	0.72 $\pm$ 0.09	0.91 $\pm$ 0.20
Salicin (5TMS)	-0.87 $\pm$ 1.48	2.87 $\pm$ 3.48	-1.54 $\pm$ 0.32	-0.42 $\pm$ 0.78
Serine (2TMS)	1.11 $\pm$ 0.28	0.89 $\pm$ 0.23	2.29 $\pm$ 0.17	1.00 $\pm$ 0.36
Serine (3TMS)	1.52 $\pm$ 0.94	0.48 $\pm$ 0.06	0.82 $\pm$ 0.11	0.61 $\pm$ 0.14
Serine (4TMS)	0.84 $\pm$ 0.26	1.16 $\pm$ 0.40	3.91 $\pm$ 0.51	1.29 $\pm$ 0.50
Serine, O-acetyl- (2TMS)	0.95 $\pm$ 0.20	1.05 $\pm$ 0.11	1.41 $\pm$ 0.19	1.78 $\pm$ 0.40
Sitosterol, β - (1TMS)	1.03 $\pm$ 0.45	0.97 $\pm$ 0.41	1.59 $\pm$ 0.65	1.10 $\pm$ 0.38
Stigmasterol (1TMS)	1.05 $\pm$ 0.48	0.95 $\pm$ 0.43	1.71 $\pm$ 0.62	1.69 $\pm$ 0.62
Succinic acid (2TMS)	0.85 $\pm$ 0.19	1.15 $\pm$ 0.29	0.86 $\pm$ 0.14	0.90 $\pm$ 0.21
Sucrose (8TMS)	0.64 $\pm$ 0.83	1.36 $\pm$ 0.24	0.44 $\pm$ 0.15	1.43 $\pm$ 0.39
Tagatose (1MEOX) (5TMS) BP	1.57 $\pm$ 0.52	0.43 $\pm$ 0.13	1.30 $\pm$ 0.95	0.66 $\pm$ 0.23
Threonic acid (4TMS)	1.08 $\pm$ 0.21	0.92 $\pm$ 0.12	0.80 $\pm$ 0.08	1.18 $\pm$ 0.28

Analyte	Normalised abundance (mean $\pm$ SEM)			
	Control 4	Control 6	ATCS 6	ATCS 16
Threonine (3TMS)	1.41 $\pm$ 0.68	0.59 $\pm$ 0.05	0.87 $\pm$ 0.10	0.73 $\pm$ 0.15
Threonine, DL- (2TMS)	1.18 $\pm$ 0.28	0.82 $\pm$ 0.18	2.29 $\pm$ 0.15	0.89 $\pm$ 0.25
Turanose (1MEOX) (8TMS) BP	0.84 $\pm$ 0.21	1.16 $\pm$ 0.17	2.12 $\pm$ 0.30	2.22 $\pm$ 0.55
Tyrosine (3TMS)	0.83 $\pm$ 0.56	1.17 $\pm$ 0.72	3.94 $\pm$ 1.12	1.64 $\pm$ 0.91
Uracil (2TMS)	0.63 $\pm$ 0.25	1.37 $\pm$ 0.37	0.94 $\pm$ 0.37	3.24 $\pm$ 0.54
Uric acid (4TMS)	1.67 $\pm$ 1.06	0.33 $\pm$ 0.21	0.74 $\pm$ 0.14	0.36 $\pm$ 0.36
Uridine (4TMS)	0.52 $\pm$ 0.15	1.48 $\pm$ 0.74	0.13 $\pm$ 0.05	1.64 $\pm$ 0.73
Valine (1TMS)	0.00 $\pm$ 0.00	2.00 $\pm$ 2.00	0.00 $\pm$ 0.00	3.59 $\pm$ 3.59
Valine (2TMS)	1.46 $\pm$ 1.01	0.54 $\pm$ 0.05	0.79 $\pm$ 0.10	1.03 $\pm$ 0.41
Xylitol (5TMS)	1.05 $\pm$ 0.14	0.95 $\pm$ 0.07	0.85 $\pm$ 0.07	0.81 $\pm$ 0.10
Xylose (1MEOX) (4TMS) MP	1.60 $\pm$ 1.10	0.40 $\pm$ 0.03	0.28 $\pm$ 0.03	0.31 $\pm$ 0.05
Xylulose-5-phosphate (1MEOX) (5TMS) BP	1.07 $\pm$ 0.30	0.93 $\pm$ 0.10	0.24 $\pm$ 0.04	1.06 $\pm$ 0.17

α -1-O-methyl-galactopyranoside peaks. Even if the β -1-O-methyl-galactopyranoside match was correct, the ratio of the two isomers was not close to one and so does not match the expected result of chemical methylation. An alternative explanation is that the tag corresponding to α -1-O-methyl-galactopyranoside was misidentified.

Other chemical derivatives found at altered concentrations in *ATCS* line 6 methanol extracts were each represent one of several different derivatives of a particular metabolite. All derivatives were compared to determine if *in vivo* levels were likely to be altered in the *ATCS* lines.

Asparagine

Asparagine [-H₂O] (2TMS) levels in *ATCS* line 6 extracts were 3.1 ± 0.5 times those of controls ($p < 0.0005$). Average asparagine (2TMS) levels were 1.6 ± 0.3 times those of control extracts. However, average asparagine (3TMS) levels were less than in control line 4 extracts. As such, it could not be concluded that *in vivo* asparagine levels in *ATCS* line 6 differed to those of the control lines.

Aspartate

Aspartic acid (3TMS) levels in control extracts varied widely. Control line 4 levels were three times those of control line 6 ($p < 0.05$). Aspartic acid (3TMS) was undetected in *ATCS* line 6, and was therefore significantly less abundant ($p < 0.0005$). However, average levels of aspartic acid (2TMS) were 0.9 ± 0.2 times control levels. The interpretation is complicated by altered ratios of the two derivatives in each line. While the 3TMS derivative was the more abundant derivative in control and *ATCS* line 16 extracts, the 3TMS derivative was the more abundant in *ATCS* line 6. As such, it is not possible to conclude that aspartate levels were lower in *ATCS* line 6.

Galactose-6-phosphate

The intensity of the second galactose-6-phosphate (1MEOX) (6TMS) peak in *ATCS* line 6 extracts was half (0.46 ± 0.07) that of the control average ($p < 0.0005$). The mean of the earlier peak was $39\% \pm 5\%$ of the controls. These results suggest a decreased *in vivo* galactose-6-phosphate content in *ATCS* line 6.

Glucose

Levels of D-glucopyranose (5TMS) in *ATCS* line 6 extracts were 13 ± 1 times the control levels ($p < 0.0005$), but average levels of the two glucose (1MEOX) (5TMS) derivatives were lower in *ATCS* line 6 extracts than controls. Therefore, it was not concluded that glucose levels in *ATCS* line 6 were different to the control lines.

Glucose-6-phosphate

Glucose-6-phosphate (6TMS) yields in *ATCS* line 6 extracts were 105 ± 28 times those of control extracts ($p < 0.0005$), but the (1MEOX) (6TMS) derivative was significantly less abundant than in control extracts, at $48\% \pm 7\%$ of control levels. The (1MEOX) (6TMS) derivative is the primary derivative of glucose-6-phosphate in each of the extracts, and is even present in *ATCS* line 6 extracts at more than 10 times the level of the (6TMS) derivative. Therefore it was not concluded that *ATCS* line 6 glucose-6-phosphate levels were increased.

Glutamine

DL-glutamine (4TMS) in *ATCS* line 6 extracts was detected at 7.1 ± 1.0 times the level in control extracts ($p < 0.0005$). Average levels of glutamine $[-H_2O]$ (2TMS) MP and DL-glutamine (3TMS) in *ATCS* line 6 extracts were 3.5 ± 0.9 and 2.5 ± 1.3 times those of control extracts, respectively. Glutamine $[-H_2O]$ (2TMS) BP abundance in *ATCS* line 6 extracts averaged 0.95 ± 0.29 times the control average. Paired student's *t* tests comparing the average values of all glutamine derivatives between *ATCS* line 6 and each of the control lines

did not indicate that glutamine was significantly more abundant in *ATCS* line 6 than either of the control lines.

Serine

Serine (2TMS) and (4TMS) derivatives were 2.3 ± 0.2 and 3.9 ± 0.5 times more abundant, respectively, in *ATCS* line 6 extracts than control line extracts ($p < 0.0005$). Serine (3TMS) intensity, by contrast, was 0.8 ± 0.1 of the average control intensity. Serine (3TMS) gave the largest peak of the three serine derivatives in all lines except *ATCS* line 6, in which the 2TMS derivative was more intense. Therefore, it could not be concluded that serine levels were altered in *ATCS* line 6.

Summary of individual metabolite changes

It was concluded that *ATCS* line 6 had increased *in vivo* ethanolamine and galactinol content, compared to control lines, while galactose-6-phosphate, glycerol-3-phosphate, *myo*-inositol-1-phosphate and xylulose-5-phosphate were all less abundant. *ATCS* line 16 had increased galactinol and uracil content, while fumarate content was reduced.

Multivariate analysis of methanol extract GC-MS data

PCA was performed on the logarithmic growth metabolome data. This identified three statistically significant principal components accounting for 60.3% of inter-sample variance (Figure 4.14a). MANOVA of principal component scores revealed significant differences between *ATCS* line 6 and every other transgenic line (controls and *ATCS* line 16; $p < 0.005$). There were no significant differences between the two control lines or *ATCS* line 16 and either of the control lines. ANOVA of each component revealed significant differences in component three, which accounted for 12.5% of inter-sample variance ($p < 0.05$). Student's *t* tests revealed highly significant differences between *ATCS* line 6 and control line 6 in scores

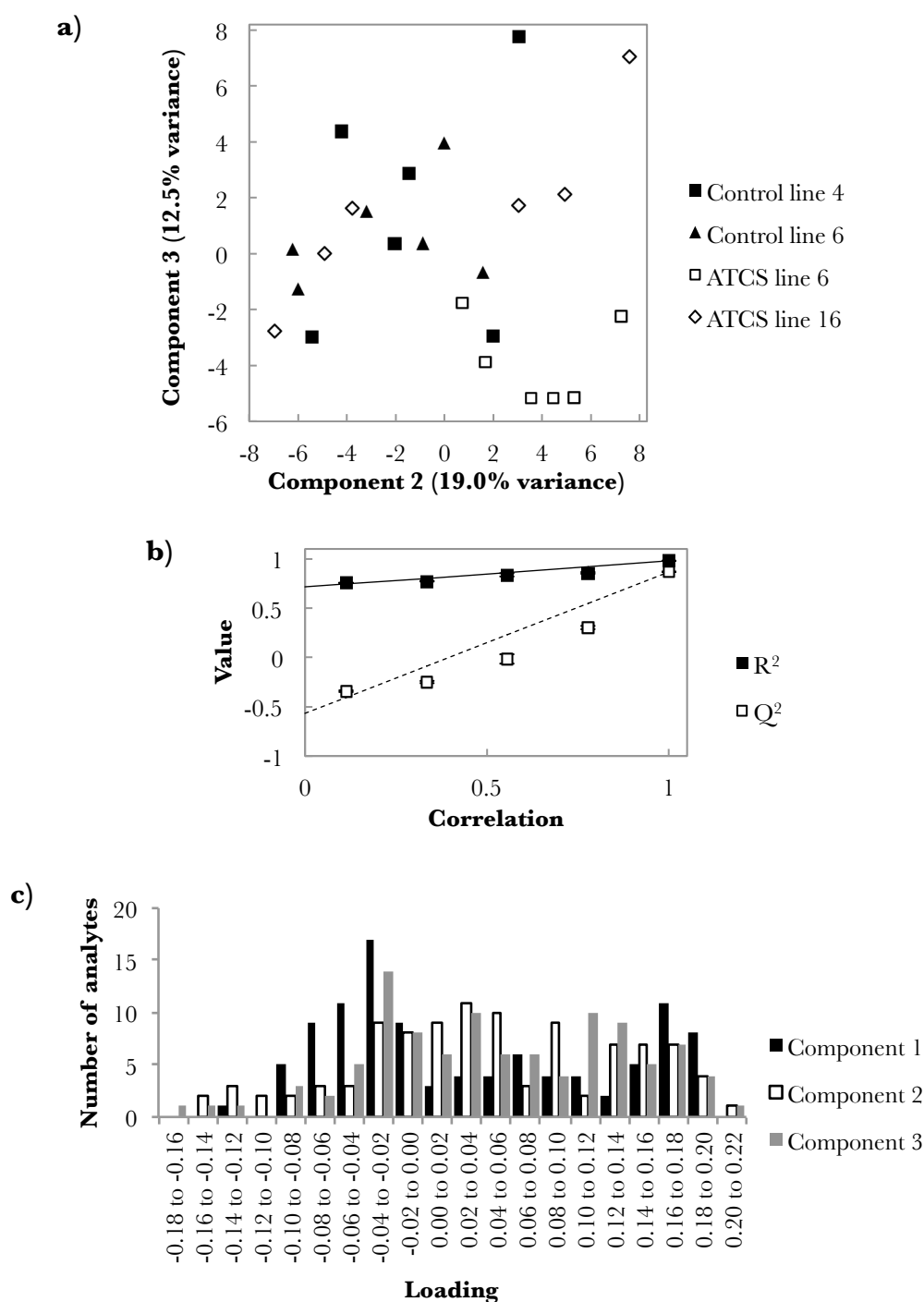


Figure 4.14. Principal component analysis of methanol-soluble metabolite GC-MS results from transgenic *ATCS* and control BY-2 cell lines undergoing logarithmic growth.

a) Scores plot of PCA components two and three, which provided greatest separation of *ATCS* line 6 from control lines. (Separation by component one is provided in Appendix 4.13.) **b)** Partial least squares projection to latent structures validation of *ATCS* line 6. Samples were randomly assigned to either “*ATCS* line 6” or “control” groups 999 times. R^2 y-intercept = 0.714, Q^2 y-intercept = -0.567. **c)** Distribution of loadings for PCA components.

for all components ($p < 0.005$). *ATCS* line 6 had significantly different scores to control line 4 for components two and three, which together accounted for 31.5% of inter-sample variance ($p < 0.05$). *ATCS* line 6 was also different to *ATCS* line 16 in its component one and three scores, accounting for 40.7% of inter-sample variance ($p < 0.01$). There were no significant differences between any component scores for any pairwise comparisons between *ATCS* line 16, control line 4 or control line 6.

Principal component one provided the greatest separation of the first replicate of control line 4 from the other experimental samples (Appendix 4.13). This sample was not greatly different from other control line 4 samples when PCA was performed on ribitol-normalised data (Appendix 4.12), raising the possibility that this measurement became an outlier due to the normalisation to alkanes. Omitting this replicate from *t* tests suggested a highly significant difference between *ATCS* line 6 and control line 4 ($p < 0.0005$). To test if inclusion of this replicate affected the significance of the results, PCA of alkane-normalised data was performed again after omitting this replicate. This still indicated significant differences between *ATCS* line 6 and the other lines. There were also significant differences between *ATCS* line 16 and the other lines based on component three scores, but this accounted for only 13.0% of inter-sample variance. There were no significant differences between the two control lines.

Loadings of the principal components were investigated to gain an insight into the types of metabolite contributing to the separation of *ATCS* line 6 from the control lines. *ATCS* line 6 samples had lower scores than control line 6 on component one, higher component two scores than either of the control lines, and lower component three scores. Table 4.3 provides a summary of the division of different metabolite types between those with negative, neutral and positive loadings for each of the principal component. Figure 4.14c provides an overview of the distribution of loadings for each principal component. Appendix 4.14 lists the loadings of

each analyte. This data suggests a general increase in soluble amino acid content of *ATCS* line 6, as 27 of the 34 analytes comprising amino acids had loadings greater than 0.05 for component two. Amino acids also made up more than half of the loadings less than -0.05 for component one. There tended to be an abundance of sugars and organic acids with loadings greater than 0.05 for components one and three, suggesting a general depletion of these metabolite groups in *ATCS* line 6.

Partial least squares projection to latent structures was performed as a supervised statistical analysis of the variation between *ATCS* line 6 and the control lines. This was done by randomly assigning samples 999 times to one of two groups, one of which initially contained *ATCS* line 6 samples, the other of which contained control samples. Jack-knifing was used to test for over-fitting of the metabolome data to each group. In each case, the explanatory quality and predictive quality of the latent variables of the simulated groupings reduced as correlation to the true grouping reduced (Figure 4.14b). This suggested that the data was not over-fitted and provided further evidence that *ATCS* line 6 was significantly different to the control lines.

4.3. Discussion

4.3.1. *ATCS* lines were consistently different to controls in their citrate synthase activities and sterol profiles

Transformation of BY-2 cells with an *Arabidopsis* mitochondrial CS gene expressed under the control of the cauliflower mosaic virus 35S promoter resulted in numerous lines with increased CS activity. It was noticed that CS activity in extracts from suspension cultures tended to be

Table 4.3. Overview of analytes, grouped by principal component loadings.

ATCS line 6 had a significantly lower component two score than the two control lines, and a significantly higher component three score. Its component one score was significantly higher than control line 6. Its component one score was significantly higher than that of control line 4 when replicate one of control line 4 was omitted. Therefore, *ATCS* line 6 would be expected to be relatively enriched for analytes with positive loadings for components one and three and negative loadings for component two. Loadings for each analyte and their categorisation are presented in Appendix 4.14.

	Total identified	Component 1 loadings			Component 2 loadings			Component 3 loadings		
		$x < -0.05$	$-0.05 < x < 0.05$	$x > 0.05$	$x < -0.05$	$-0.05 < x < 0.05$	$x > 0.05$	$x < -0.05$	$-0.05 < x < 0.05$	$x > 0.05$
Amino acids	35	12	10	13	1	7	27	5	21	9
Organic acids	25	2	8	15	5	13	7	1	13	11
Sterols	5	0	5	0	0	2	3	0	0	5
Sugars	20	6	4	10	2	14	4	2	7	11
Triacylglycerol components	10	0	6	4	3	6	1	0	1	9
Other metabolites	8	2	6	0	2	3	3	3	2	3
Total	103	22	39	42	13	45	45	11	44	48

metabolism not specifically related to the activity of the *35S* promoter were responsible for the increased activity on solid medium compared to in suspension. Other possibilities include differences in population growth and heterotrophy in suspension (Kovarík et al., 2012). It should be noted that the activity of callus extracts represent single measurements, so within line comparisons between callus and suspension activity lack statistical robustness. However, CS activity of lines that were also grown in suspension was approximately 50% higher in callus extracts than suspension culture extracts.

The two lines with highest CS activity were analysed in detail. *ATCS* line 6 had 2.6 times the extractable CS activity of control lines transformed with an *mCherry* construct. *ATCS* line 6 also had a lower growth rate and subtle but significantly different changes to its primary metabolome compared to control lines. It exhibited increased ethanolamine and galactinol content, while galactose-6-phosphate, glycerol-3-phosphate, *myo*-inositol-1-phosphate and xylulose-5-phosphate levels were reduced. *ATCS* line 6 also had a slight increase in total sterols. This increase was primarily the result of an accumulation of end-of-pathway sterols, specifically cholesterol, stigmasterol and campesterol, as well as the near-end sitosterol. Other sterols were significantly decreased in this line. Notably, extractable acetyl-CoA and citrate (as well as excreted citrate), and oxygen consumption were not affected by the increased CS activity.

ATCS line 16, which had extractable CS activity 2.1 times that of controls, was mainly characterised by a slightly increased oxygen uptake and increases in the abundance of stigmasterol and sitosterol. These increases were similar to those of *ATCS* line 6 though less pronounced, as were the relative decreases in 24-methylene cholesterol and fucosterol. As a result, the sterol profile of this line was similar to that of *ATCS* line 6, but total sterol levels were no different to controls. The methanol-soluble metabolome, as measured using a broad spectrum GC-MS approach, was also more similar to control lines than the *ATCS* line 6

metabolome was, with the only significant differences being increases in galactinol and uracil and a decrease in fumarate content.

Thus, the only consistent observed changes correlating with increased CS activity were increased levels of end product sterols, seemingly at the expense of intermediate sterols, and increases in galactinol.

4.3.2. Specific alterations to the sterol profile differ from published manipulations to early steps in sterol biosynthesis.

The shift in the sterol profile towards greater abundance of end-of-pathway sterols at the expense of earlier pathway intermediates was identified in both *ATCS* lines and suggests a change in sterol biosynthetic flux. This shift would be expected if end-of-pathway sterols were quickly conjugated and sequestered away from sterol biosynthetic reactions. Such a phenotype was observed in *Arabidopsis* with increased stigmasterol levels resulting from over-expression of a sterol C-22 desaturase responsible for the conversion of sitosterol to stigmasterol (Arnqvist et al., 2008). However, there were no differences between the proportion of total sterols calculated to be conjugated in *ATCS* lines compared to controls, and the proportion of end product sterols in the conjugated sterol profile was no different in any line to the proportion of end product sterols in the free sterol profile. An alternative explanation may be that the end product sterols are being quickly inserted into membranes and are effectively removed from the metabolic pool in this manner. Analyses of lipid composition could have provided evidence to support this hypothesis.

Tobacco seeds (Holmberg et al., 2002; Holmberg et al., 2003) and Arabidopsis leaves (Beste et al., 2011) with increased activity of sterol C-24 methyltransferase (SMT1), the enzyme responsible for methylating cycloartenol in the first committed step of the biosynthesis of stigmasterol and campesterol, had highest increases in the yields of sterols proximal to that reaction. Similarly, the largest relative increases were discovered in early pathway sterols in tobacco leaves (Gondet et al., 1994), seeds (Harker et al., 2003b; Holmberg et al., 2003) and cotyledon-derived calli (Schaller et al., 1995) with increased activity of HMGR, the primary control point for mevalonate pathway flux. Combining SMT1 and HMGR activity increases gave greater yields of all sterols downstream of cycloartenol than either enzyme manipulation on its own, but end product sterols as a proportion of total sterols was still lower than in WT plants (Holmberg et al., 2003). Over-expression of C-22 desaturases in Arabidopsis did increase stigmasterol yields (at the expense of sitosterol), but levels of intermediate sterols were relatively unaltered (Arnqvist et al., 2008).

The exception to these increases in intermediate sterols has been soybean (*Glycine max*) seeds, in which seed-specific overexpression of SMT1 or truncated HMGR genes resulted in increased proportions of sitosterol, campesterol and stigmasterol at the expense of cycloartenol and 24-methylene cycloartenol (Neelakandan et al., 2012). This could be indicative of regulation of the downstream steps of sterol biosynthesis in some systems, such as soybean seeds and BY-2 cells, being less tightly controlled than in other systems like tobacco and Arabidopsis plants. Alternatively, the increased proportions of end product sterols in *ATCS*-overexpressing BY-2 cells may be indicative of an increased supply of acetyl-CoA causing a more co-ordinated up-regulation of the sterol biosynthetic pathway as a whole than when steps within isoprenoid or sterol biosynthesis are targeted directly.

4.3.3. Measurements of total citrate and acetyl-CoA may not reflect their availability to sterol biosynthesis

The assays of citrate and acetyl-CoA are incapable of differentiating between different pools of the metabolites. Citrate in plant cells is primarily located in the vacuole (Yamaki, 1984; Echeverria and Valich, 1988; Gout et al., 1993; Farré et al., 2001). It is possible for a large and variable vacuolar citrate pool to mask a large relative increase in cytosolic citrate, or for the relative proportions of citrate in each subcellular compartment to be altered without affecting the total cellular content. Similarly, mitochondrial, plastidial and peroxisomal acetyl-CoA could mask increases in cytosolic acetyl-CoA content.

Cytosolic acetyl-CoA cannot be measured directly. However, cytosolic [^{13}C]citrate may be quantified by nuclear magnetic resonance (NMR) spectroscopy following the addition of paramagnetic Mn^{2+} to first quench the vacuolar citrate signal (Gout et al., 1993). This is a complicated and time-consuming experiment that has not been tested on BY-2 cells. It involves culturing cells up to metabolic steady state in medium containing [$^{13}\text{C}_6$]glucose, creating protoplasts and performing *in vivo* ^{13}C -NMR on protoplasts perfused with an oxygenated Mn^{2+} -containing isotonic medium. While it might have given an indication of whether steady state citrate levels were altered in *ATCS* lines, this experiment would not determine if flux through citrate was increased. Metabolic flux analysis, described in the following chapter, is able to provide that information. For this reason specific attempts were not made to quantify steady state cytosolic citrate or acetyl-CoA content.

4.3.4. With the exception of sterols, the metabolome did not consistently change in lines with increased citrate synthase levels

With the exception of a tripling of galactinol content, there were no significant changes identified in one *ATCS* line by a broad-spectrum metabolomic approach that matched changes in the other *ATCS* line. There were also no changes in metabolites that were metabolically closely related to each other. Galactinol has been implicated in tolerance to cold and drought stresses (Taji et al., 2002; Rabara et al., 2013). It was interesting to note that fumarate levels were reduced in *ATCS* line 16, given the proximity of fumarate to citrate in the TCA cycle. However, it is not clear if this represents a reduction in mitochondrial fumarate levels. Also, this line had a less pronounced phenotype overall than *ATCS* line 6, which suggests that the decreased fumarate was not relevant to the overall phenotype.

4.3.5. Conclusions

The increase in end product sterols may affect membrane properties in the *ATCS* lines, given the superior membrane ordering effects of campesterol and sitosterol compared to other sterols (Schuler et al., 1990; Schuler et al., 1991; Krajewski-Bertrand et al., 1992). It is interesting to note that, despite only minor differences in the CS content of each *ATCS* line, the line with highest CS content (*ATCS* line 6) had a near doubling in the total level of these sterols, a significantly reduced growth rate and an altered metabolome, while the other *ATCS* line (16) analysed in detail had only a small increase in sitosterol content and no increase in campesterol content, and, by other measurements, appeared little different to control lines. This raises the question of whether an altered membrane sterol profile in *ATCS* line 6 was in any way responsible for the other phenotypic differences observed in this line, if altered

membrane properties were required as a result of other phenotypic differences in the line, or if the sterol profile shifted to favour the synthesis of end of pathway sterols due to increased substrate availability.

This final option is the simplest and it was originally hoped that increased CS activity would increase the supply of precursors for sterol biosynthesis, with the effect of increasing sterol biosynthetic flux. This would suggest that the supply of acetyl-CoA is a significant constraint on sterol biosynthetic flux, and that CS has a significantly positive control coefficient for that flux.

The results presented here demonstrate the relevance of targeted over-expression of primary metabolism enzymes for increasing yields of targeted secondary metabolites, as has already been demonstrated in bacteria (Choi et al., 2010), and, more generally, that consideration of the entire metabolic network when engineering plants for altered yields of specific metabolites can result in desirable phenotypes. The following chapter investigates the primary metabolic fluxes of the *ATCS* lines and will help to elucidate the mechanism by which end-product sterol levels are increased and the differences between the two *ATCS* lines.

Chapter 5. Metabolic flux analysis of wild-type and transgenic *ATCS* and control BY-2 cell lines

5.1 Introduction

5.1.1. Objectives

Increased flux through citrate synthase (CS) is required for an increase in CS abundance to increase acetyl-CoA availability for the mevalonate pathway. The absence of altered acetyl-CoA or citrate in observations presented in Chapter 4 could conceal a change in flux through CS, because fluxes into a metabolite can vary without affecting the steady state concentration of that metabolite if the fluxes out of the metabolite vary by the same degree. The changes in sterol profiles suggest that some degree of flux rearrangement occurred in those lines with increased CS activity. It is expected that any changes in the transgenic cell lines with increased CS activity would be most pronounced in tricarboxylic acid (TCA) cycle flux.

The aim of this chapter is to determine the effects of increased CS activity on metabolic fluxes in tobacco (*Nicotiana tabacum*) Bright Yellow 2 (BY-2) cells. This is achieved by first determining when cultures are at isotopic steady state, i.e. when the isotopic composition of each metabolite is unchanging while cultures are being fed a specific ratio of stable isotopes (e.g. $^{13}\text{C}:^{12}\text{C}$). Biomass synthesis is then assessed by following the redistribution of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$. This is followed by analysis by gas chromatography coupled to mass spectrometry (GC-MS) of the redistribution of ^{13}C from ^{13}C -glucose into various metabolites, and quantification of the metabolic fluxes of primary metabolism using 13C-FLUX software. The results lead to a greater appreciation of the effects of increased CS activity on BY-2 metabolism.

5.1.2. Acquiring information on metabolic fluxes by steady-state metabolic flux analysis

Steady-state MFA provides a viable means of experimentally determining metabolic fluxes. Cultures are usually fed ^{13}C -labelled substrates. The ^{13}C label becomes redistributed throughout the network in a manner that is dependent on the relative fluxes through different reactions. This redistribution is manifested in specific metabolite isomers known as isotopomers, which are isotopically labelled at specific carbons positions. Knowledge of the relative proportions of different isotopomers and metabolic reaction stoichiometries can be used to determine the metabolic fluxes (Figure 5.1). Due to the complexity of the metabolic network, computational tools are required to determine fluxes based on isotopomer data after first defining the metabolic reaction stoichiometries as a mathematical model.

Isotopomer data is gathered while cultures are in an isotopic and metabolic steady state (Roscher et al., 2000), where carbon flux into metabolic intermediates is balanced by efflux out of them. Data is collected using mass spectrometry-based methods (van Winden et al., 2005; Schwender et al., 2006), nuclear magnetic resonance (NMR) spectroscopy (Rontein et al., 2002; Sriram et al., 2004; Ettenhuber et al., 2005; Alonso et al., 2007b; Alonso et al., 2007c; Williams et al., 2008; Masakapalli et al., 2010) or a combination of the two (Schwender et al., 2003; Alonso et al., 2007a; Alonso et al., 2010; Alonso et al., 2011; Masakapalli et al., 2013). Isotopomer data are added to the model along with some known reaction rates, which can be determined from the rate of biomass accumulation. Various pieces of software may be used to determine which flux values provide the best description of the isotopomer, biomass and model structure data provided (Wiechert et al., 2001; Sriram et al., 2004; Ettenhuber et al., 2005; Zamboni et al., 2005; Srouf et al., 2011; Sokol et al., 2012; Weitzel et al., 2013).

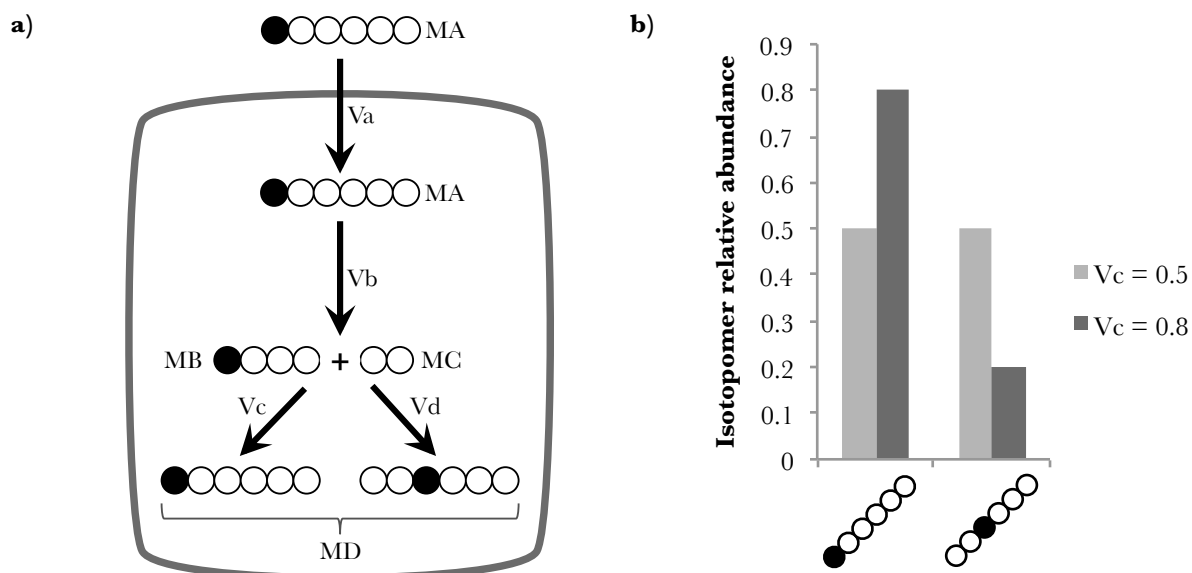


Figure 5.1. Steady-state metabolic flux analysis of a simple hypothetical network.

a) A simple metabolic network involving a six-carbon substrate (MA) isotopically labelled at C1 being imported into a cell (by flux V_a) and split into a four-carbon compound (MB) and a two-carbon compound (MC) (V_b). A new six-carbon compound (MD) is created by either adding MC to MB in the same order as in MA (V_c) or by recombining in the order MC-MB (V_d). V_c and V_d are indistinguishable when an unlabelled substrate is metabolised, but metabolism of a substrate labelled at C1 leads to different isotopomers of MD being created by V_c and V_d . **b)** The graph indicates the relative proportions of the two MD isotopomers that occur when flux through V_c is either 0.5 or 0.8 relative to an uptake flux (V_a) of 1. (In this example MX indicates a metabolite and V_x indicates a flux. A representation of this pathway with example mass isotopomer measurements for $V_c = 0.8$ is presented in Appendix 5.1 in 13C-FLUX format.)

Steady-state MFA is particularly useful for determining fluxes in complex networks where carbon can flow by a variety of different routes to achieve the same products. Hence it is relevant to primary metabolism. However, the nature of the label redistribution necessitates that the model provides a simplified overview of the metabolic network under examination. Where labelling information is unable to distinguish between subcellular pools of the same metabolite, they are treated as a single pool. Similarly, labelling data only provides information on reactions where carbon rearrangement occurs, with the consequence that several reactions may need to be combined into a single step in the model. For instance, it is often not possible to distinguish between the conversion of α -ketoglutarate to succinate by the TCA cycle and by the γ -amino-butyrate (GABA) shunt, so these fluxes are usually combined into a single step attributed to the TCA cycle (Kruger et al., 2012). Also branches of metabolism with no carbon rearrangements must be ignored. Thus much of secondary metabolism is omitted.

5.1.3. Use of steady-state metabolic flux analysis in the elucidation of plant metabolism

Steady-state MFA has provided intriguing insights into the operation of metabolism in plants. MFA of heterotrophic *Arabidopsis* (*Arabidopsis thaliana*) cell cultures has demonstrated the robustness of the primary metabolic network to increases in O₂ concentration and shown that changes in steady state metabolite composition can occur without significant changes in the flux distribution (Williams et al., 2008). It has also revealed the role of the plastid in starch biosynthesis and the changes to primary metabolism caused by oxygen limitation in maize (*Zea mays*) root tips (Alonso et al., 2007b; Alonso et al., 2007c), as well as the increased TCA

cycle flux in the *Arabidopsis wrinkled1* mutant, which is deficient in lipid biosynthesis (Lonien and Schwender, 2009).

Steady-state MFA of heterotrophic plant cell cultures has been conducted successfully (Rontein et al., 2002; Williams et al., 2008; Masakapalli et al., 2010; Masakapalli et al., 2013). It has also been successfully implemented in analyses of transgenic plant lines (Lonien and Schwender, 2009). However, this is, to my knowledge, the first example of steady-state MFA of a tobacco cell culture and of transgenic plant lines over-expressing the gene for a single enzyme of primary metabolism.

5.1.4. Experimental approach

The experimental approach to the chapter involved the following:

1. Establishment of metabolic and isotopic steady state: Cultures must achieve an isotopic and metabolic steady state in order for MFA to give an accurate model of metabolic flux (Ratcliffe and Shachar-Hill, 2006). Metabolic steady state has already been determined for wild-type (WT) (Chapter 3.2.2.4) and transgenic *ATCS* and control lines (Chapter 4.2.7.1). WT, the transgenic control lines, and *ATCS* line 16 were judged to be in a metabolic steady state between four and five days after subculture. *ATCS* line 6 was at a metabolic steady state between four and six days.
2. Definition of the model structure. This involves deciding which metabolic pathways and reactions should be included in the model and presenting reaction stoichiometries and fluxes in the 13C-FLUX fibl file format.

3. Calculation of biomass output fluxes. This is done by analysing the redistribution of ^{14}C from a ^{14}C -labelled substrate into various metabolite groups and quantifying different metabolites within these groups using GC-MS of unlabelled samples that are processed in parallel.
4. Performing the MFA feeding experiment using $[^{13}\text{C}]$ glucose at isotopic and metabolic steady state and extracting mass isotopomer data from GC-MS analyses of different metabolites. These data are then refined by correcting for the contribution of unlabelled metabolites and by omitting unreliable or noisy measurements.
5. Initial MFA using 13C-FLUX to identify any further unreliable mass isotopomer data based on its agreement with predictions made by 13C-FLUX. This is so that such data may be omitted from later analyses. The model structure is also verified at this stage by comparing the labelling patterns of metabolites with the expected patterns arising from different possible metabolic routes. Biomass output fluxes are constrained based on the initial model runs.
6. Further MFA using Monte Carlo simulation (MCS) of the mass isotopomer data. This is done to gain an insight into the determinability of each flux and to get average free flux values with which to provide the model such that a best-fit solution can then be achieved with a final 13C-FLUX run.
7. Statistical analyses of each flux in each metabolic model using linear and non-linear methods.
8. Validation of models by making comparisons with experimental data, comparisons between genotypes of fluxes and assessing coenzyme provision.

5.2. Results

5.2.1. Determination of isotopic steady state

Isotopic steady state was determined by sub-culturing BY-2 cells into medium containing 20 mol% [$^{13}\text{C}_6$]glucose. The ^{13}C molar ratio of this [$^{13}\text{C}_6$]glucose is approximately 99 mol% equating to approximately 94 mol% of the $^{13}\text{C}_6$ isotopomer. Feeding of ^{13}C -labelled substrates to higher plant cells has been demonstrated to have no appreciable effect on intracellular fluxes (Kruger et al., 2007). Cultures were harvested at 24 h intervals during the logarithmic phase of growth. Fractional enrichment of ^{13}C was then measured in soluble amino acids and protein hydrolysate using GC-MS.

Glucose content of culture medium

A preliminary analysis of WT cell culture filtrate (CCF) was performed to determine whether isotopic steady state was likely to be reached before depletion of glucose from the cell medium. The presence of glucose in the CCF throughout the duration of isotopic steady state analysis was verified by GC-MS. The $^{13}\text{C}_6$ isotopomer was at 18.0 ± 0.2 mol% (mean $\pm$ standard error (SEM)) throughout the experiment suggesting that the glucose present in the medium was not endogenously synthesised by the WT BY-2 cells, in which case a greater degree of redistribution of the ^{13}C would be expected and the proportion of the $^{13}\text{C}_6$ isotopomer would decrease. The $^{13}\text{C}_6$ isotopomer is slightly less abundant than the prediction of 18.8 mol%. This is probably due to dilution by unlabelled glucose carried over from the seeding culture during subculture.

Isotopic steady state of amino acids

Cultures are said to be at isotopic steady state when ^{13}C fractional enrichment of metabolites is unchanging. This was assessed by 1) comparing the ^{13}C fractional enrichment of soluble

amino acids and protein hydrolysate to that of glucose in CCF, 2) evaluating ^{13}C fractional enrichment of soluble amino acids and protein hydrolysate over time, and 3) comparing ^{13}C fractional enrichment in metabolites with slow turnover (protein hydrolysate) with metabolites with high turnover (soluble amino acids).

Amino acid-*t*-butyldimethylsilyl (TBDMS) fragments arising from GC-MS of methanol extracts and protein hydrolysate were identified by comparison with a library of amino acid standard TBDMS ethers created previously in our laboratory by Mr Pedro Bota. Mass isotopomer data was extracted using a MATLAB tool (Wahl et al., 2004) from abundances of fragments with different mass/charge (m/z) ratios averaged across the GC-MS peak after baseline-correction. Fragments were then assessed for measurement reliability by determining ^{13}C fractional enrichment in cultures grown in medium containing glucose with natural ^{13}C abundance (0.0113 mol%). These cultures were grown in parallel with cultures grown in 20 mol% [$^{13}\text{C}_6$]glucose medium. A fragment was considered reliable if its measured ^{13}C fractional enrichment in unlabelled samples was at 1.13 ± 3 mol% and if it was identified in at least three out of four replicates of each dataset being compared. Fragments with overlapping m/z ratios were ignored.

Wild-type

Measurements of ^{13}C fractional enrichment in soluble amino acids extracted from WT cultures did not differ significantly between four and five days after subculture (Figure 5.2a, Appendix 5.2), with ^{13}C fractional abundance being approximately 0.20 (0.201 ± 0.012 at four days; 0.198 ± 0.012 at five days). The similarity in fractional ^{13}C enrichment indicated that the soluble amino acid pool was at isotopic steady state by four days after subculture. However, most fragments did not reach the expected ^{13}C content of 0.213 ± 0.003 (the measured ^{13}C fractional enrichment of CCF glucose, mean $\pm$ SEM), suggesting that

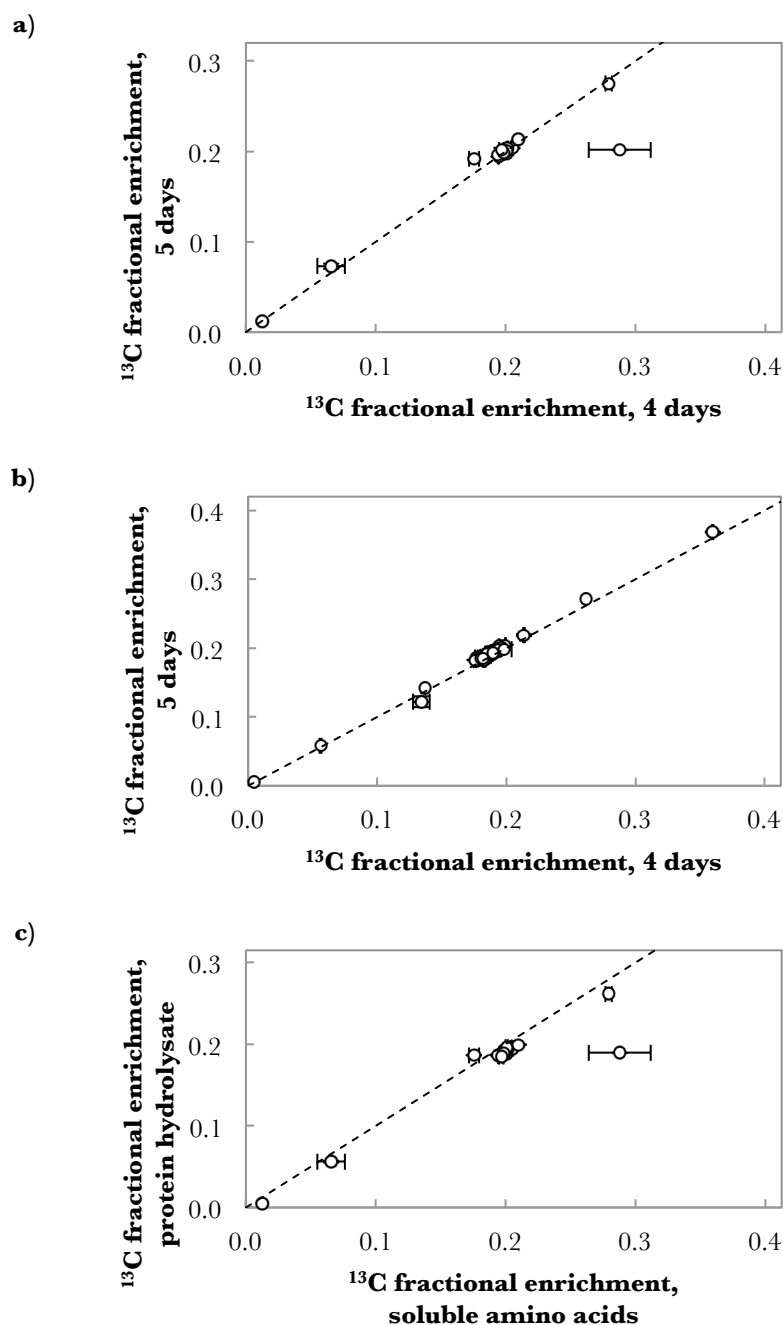


Figure 5.2. Determination of isotopic steady state in wild-type BY-2 cell suspension cultures.

Scatter charts indicating fractional enrichment of ^{13}C in various amino acid fragments determined from GC-MS data from cultures fed with 20 mol% $[^{13}\text{C}_6]\text{glucose}$. **a)** Soluble amino acids at four and five days. **b)** Protein hydrolysate at four and five days. **c)** Soluble amino acids and protein hydrolysate at four days. Trendlines indicate $y=x$. At complete isotopic steady state all data-points should fall on the line of $y=x$. Data-points represent the mean of four replicates $\pm$ SEM and are listed in Appendix 5.2.

pre-existing unlabelled pools of soluble amino acids and proteins contribute to a lower steady state ^{13}C fractional enrichment.

Slow turnover of proteins means that amino acids in protein hydrolysate may not reach isotopic steady state while the culture is in a metabolic steady state (Williams et al., 2008; Masakapalli et al., 2013). However, pools of unlabelled metabolites may be taken into account in flux models by including unlabelled metabolites as additional inputs (Lonien and Schwender, 2009; Williams et al., 2010; Kruger et al., 2012) or by mathematical adjustment of isotopomer abundances (Masakapalli et al., 2013). ^{13}C fractional enrichment in protein hydrolysate increased significantly between four and five days in 27 out of 32 comparisons ($p < 0.05$, student's t tests, Figure 5.2b). This indicates that the protein-derived amino acids were not at isotopic steady state after four days. However, no increase was greater than 0.8 mol% between the two days, suggesting that proteins were close to isotopic steady state.

The majority of fragments considered had greater ^{13}C fractional enrichment in soluble amino acids than protein hydrolysate at four days and five days ($p < 0.05$, student's t tests, Figure 5.2c). However, the difference in reliable fragments between the two pools was only 0.9 ± 0.1 mol% at four days and 0.6 ± 0.1 mol% at five days.

Thus, it was concluded that isotopic steady state of soluble amino acids was reached by four days. Protein hydrolysate did not reach isotopic steady state while the cultures were at metabolic steady state but did reach a near steady state. As such, it was decided that cultures between four and five days old would be appropriate for use in modelling following correction of protein hydrolysate data for unlabelled pools of amino acids.

Transgenic lines

By the methods outlined for the WT, control (*mCherry*) lines 4 and 6 and *ATCS* line 16 were considered to be sufficiently close to an isotopic steady state by four days after subculturing (Figure 5.3a, 5.3b and 5.3d). *ATCS* line 6 required an additional day's growth (Figure 5.3c) due to the slower growth identified in Chapter 4.2.3. Appendix 5.3 contains fractional enrichment data for amino acids fragments from cultures at isotopic steady state for each transgenic line.

There were significant increases in ^{13}C fractional enrichment in most amino acid fragments, whether soluble or protein hydrolysate, in each line between the day at which the line was deemed close enough to isotopic steady state and the following day ($p < 0.05$, student's t tests). ^{13}C fractional enrichment in soluble amino acids in each of these days was also, in most cases, significantly greater than in protein hydrolysate. However, differences between consecutive days in ^{13}C fractional enrichment of soluble amino acids were generally of less than 0.5 mol%, differences between ^{13}C fractional enrichment in protein hydrolysate between consecutive days were generally of less than 1 mol%, and differences between ^{13}C fractional enrichment in soluble amino acids and protein hydrolysate at each time-point were also of less than 1 mol%. Furthermore, ^{13}C fractional enrichment in protein hydrolysate was generally greater than 18 mol% at these first time-points. The consistent exception to this was methionine, which typically had ^{13}C fractional enrichment of 17-18 mol% at four days in control lines and *ATCS* line 16, and at five days in *ATCS* line 6. However, there may be a contribution of unlabelled carbon from methylene-tetrahydrofolate (MTHF) (Arora et al., 1985), which supplies one carbon to homocysteine to form methionine (Amir, 2010).

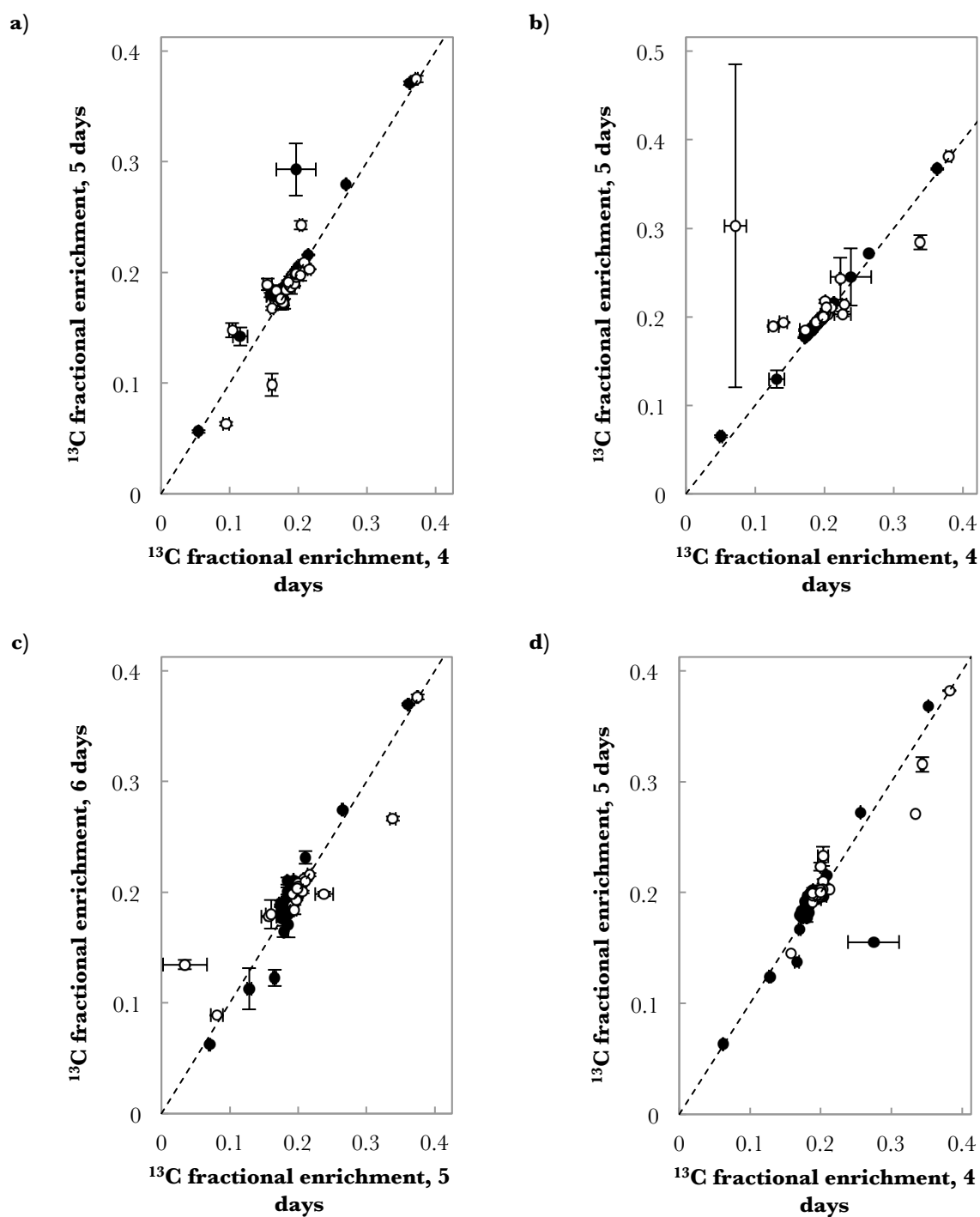


Figure 5.3. Determination of isotopic steady state in transgenic *ATCS* and control *BY-2* cell suspension cultures.

Scatter charts indicating fractional enrichment of ^{13}C in various amino acid fragments determined from GC-MS data from cultures fed with 20 mol% $[^{13}\text{C}_6]\text{glucose}$. Trendlines indicate $y=x$. At isotopic steady state data-points should fall on the line of $y=x$, and protein hydrolysate data is expected to overlap that of soluble amino acids. Data-points represent the mean of four replicates $\pm$ SEM and are listed in Appendix 5.3. $\circ$) Soluble amino acids, $\bullet$) protein hydrolysate. **a)** Control line 4. **b)** Control line 6. **c)** *ATCS* line 6. **d)** *ATCS* line 16.

5.2.2. BY-2 metabolic model design

¹³C-FLUX (Wiechert et al., 2001) was used for steady-state MFA. Detailed methodology was based on recommendations by Masakapalli (2011). The ¹³C-FLUX input (ftbl) file defines the relationship between carbon atoms in the substrates and products of each step in the metabolic network such that isotopomeric information about the organism's substrate and several of its metabolites can be used to deduce the underlying fluxes. (See Appendix 5.1 and Figure 5.1 for a simple example of this.) ¹³C-FLUX provides optimal flux values for the model by randomly assigning values to specified free fluxes and minimising the sum of squared differences (residuum) between the simulated measurements and those provided by the user. ¹³C-FLUX provides net and exchange flux values for each reaction. These are defined as follows, where $V_{forward}$ and $V_{backward}$ refer to forward flux and backward flux, respectively:

$$Net\ flux\ (V^{net}) = V_{forward} - V_{backward}$$

$$Exchange\ flux\ (V^{xch[0-1]}) = \frac{\text{minimum}(V_{forward}, V_{backward})}{1 + \text{minimum}(V_{forward}, V_{backward})}$$

It should be noted that net fluxes can be scaled to any value, but exchange fluxes are always between 0 and 0.99, with 0 representing irreversibility and 0.99 representing complete reversibility.

The model of BY-2 suspension culture steady state metabolic fluxes took reaction stoichiometries from a published Arabidopsis model (Masakapalli et al., 2010). Glycolysis and oxidative pentose phosphate pathway (PPP) steps were duplicated in cytosolic and plastidial compartments, and the non-oxidative PPP was restricted to the plastid. Proxy reactions were

included to account for unlabelled pools of arginine, glycine, histidine, methionine and serine, which have carbon contributions from exogenous (unlabelled) CO₂ or MTHF.

The mevalonate isoprenoid biosynthesis pathway was added to the model, culminating in the biosynthesis of sterols, the primary product of the mevalonate pathway in BY-2 cells (Hartmann and Bach, 2001). Also, each flux was duplicated in the model to allow two feeding strategies (20% [¹³C₆]glucose and [1-¹³C]glucose) to be used within the same model. This approach leads to smaller flux deviations than when a single feeding strategy is used (Masakapalli et al., 2010). Duplicate fluxes were constrained to carry identical flux. This model structure can be seen in 13C-FLUX format in Appendix 5.4.

5.2.3. Quantification of biomass constraints for modelling

Determination of linear uptake and redistribution of ¹⁴C from [U-¹⁴C]glucose by wild-type BY-2 cultures

Biomass output fluxes can be determined by analysing the redistribution of ¹⁴C from ¹⁴C-labelled substrates (Harrison and Kruger, 2008). Redistribution of ¹⁴C from [U-¹⁴C]glucose into CO₂, organic acids, soluble amino acids, sugars, protein, starch, cell wall, lipid and ethanol was measured in WT cultures at 12 h and 24 h after supplementation of cell cultures in early metabolic steady state. ¹⁴C in most fractions increased linearly between 12 h and 24 h (Figure 5.4a; Table 5.1). With the exception of organic acids in the CCF ($p < 0.01$, student's t test), the proportions of total metabolised ¹⁴C in each component did not alter over the course of the experiment. Radioactivity of the CCF dropped linearly over 24 h to 58% $\pm$ 3% of the initial amount, indicating linear uptake of [U-¹⁴C]glucose (mean $\pm$ SEM, four

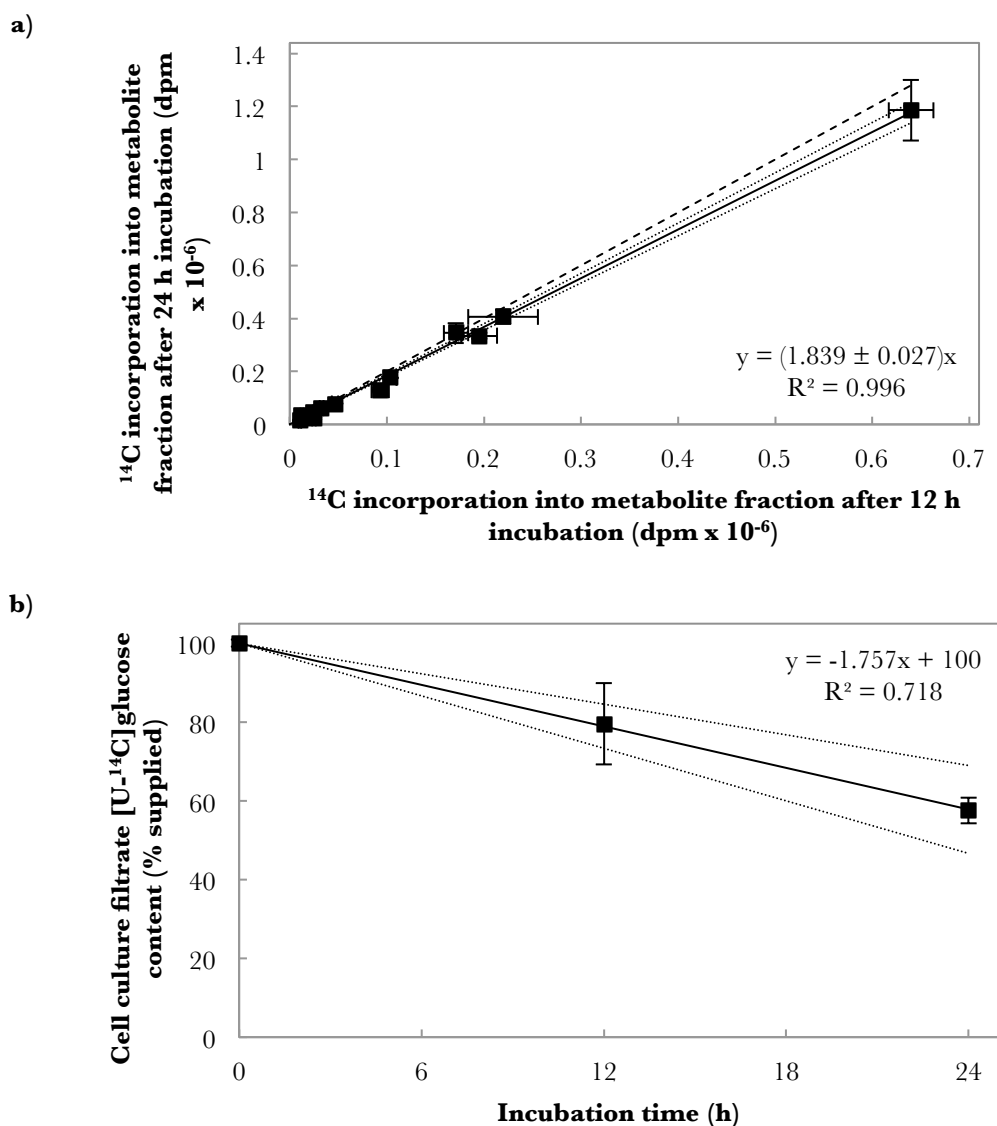


Figure 5.4. Determination of linear uptake and redistribution of [U- ^{14}C]glucose over 24 h in wild-type BY-2 cell cultures at metabolic steady state.

a) Radioactivity in most metabolite fractions approximately doubled between 12 h and 24 h. The identity of each fraction is detailed in Table 5.1. **b)** Radioactivity was measured in aliquots from cell culture at 12 h and 24 h after incubation with [U- ^{14}C]glucose. Measurements at 0h are calculated from the measured radioactivity of 2 μl of the [U- ^{14}C]glucose. In each graph, error bars represent SEM of four sample (biological) replicates, solid lines represent best fits and dotted lines indicate 95% confidence intervals determined by linear regression analysis. The dashed line in a) indicates the expected trend, $y = 2x$.

Table 5.1. Redistribution of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$ in wild-type BY-2 cell suspension cultures in metabolic steady state.

Redistribution of ^{14}C was measured by scintillation counting after fractionation of cultures fed with 185 kBq $[\text{U-}^{14}\text{C}]\text{glucose}$. The proportions of ^{14}C in each fraction were the same at 12 h and 24 h, with the exception of CCF organic acids ($p < 0.01$, student's t test). Recovery was calculated by dividing total ^{14}C recovered by total ^{14}C uptake. Total ^{14}C uptake was the sum of fractions (CO_2 , CCF organic acids, CCF protein, ethanol, chloroform-soluble (lipids), methanol-soluble and methanol-insoluble), whereas total ^{14}C recovered was the sum of sub-fractions (CO_2 , CCF organic acids, CCF protein, ethanol, chloroform-soluble (lipids), sugars, amino acids, organic acids, starch, protein, digestible cell wall and indigestible cell wall). Values are disintegrations per minute means of four biological replicates $\pm$ SEM. Percentages of total ^{14}C uptake are in parentheses.

Metabolic fraction	Radioactivity	
	12 h	24 h
CO_2^1	639,994 $\pm$ 23,000 (38.5 $\pm$ 1.6)	1,185,987 $\pm$ 114,522 (38.3 $\pm$ 2.4)
CCF organic acids¹	24,952 $\pm$ 1,967 (1.5 $\pm$ 0.2)	22,380 $\pm$ 2,660 (0.7 $\pm$ 0.1)
CCF protein¹	12,626 $\pm$ 1,495 (0.8 $\pm$ 0.1)	33,974 $\pm$ 5,132 (1.1 $\pm$ 0.2)
Ethanol¹	10,390 $\pm$ 1,395 (0.6 $\pm$ 0.1)	14,206 $\pm$ 2,244 (0.5 $\pm$ 0.1)
Chloroform-soluble (lipids)¹	32,994 $\pm$ 1,893 (2.0 $\pm$ 0.2)	60,640 $\pm$ 3,332 (2.0 $\pm$ 0.1)
Methanol-soluble	622,041 $\pm$ 94,937 (36.6 $\pm$ 3.2)	1,131,749 $\pm$ 63,011 (36.7 $\pm$ 1.6)
Neutral fraction (Sugars)¹	195,159 $\pm$ 18,560 (14.0 $\pm$ 0.8)	334,510 $\pm$ 11,676 (14.1 $\pm$ 0.7)
Basic fraction (Amino acids)¹	219,766 $\pm$ 35,819 (15.8 $\pm$ 2.2)	406,096 $\pm$ 26,705 (17.1 $\pm$ 1.3)
Acidic fraction (Organic acids)¹	93,138 $\pm$ 9,155 (6.7 $\pm$ 0.5)	128,882 $\pm$ 8,925 (5.4 $\pm$ 0.4)
Methanol-insoluble	333,197 $\pm$ 18,402 (20.0 $\pm$ 1.4)	642,148 $\pm$ 38,517 (20.8 $\pm$ 0.9)
Neutral fraction (Starch)¹	46,674 $\pm$ 3,076 (2.6 $\pm$ 0.1)	78,407 $\pm$ 690 (2.5 $\pm$ 0.1)
Basic fraction (Protein)¹	103,784 $\pm$ 6,403 (5.8 $\pm$ 0.3)	175,870 $\pm$ 1,193 (5.7 $\pm$ 0.2)
Acidic fraction (Digestible cell wall)¹	24,510 $\pm$ 402 (1.4 $\pm$ 0.1)	45,608 $\pm$ 1,004 (1.5 $\pm$ 0.1)
Indigestible cell wall¹	171,126 $\pm$ 12,574 (10.3 $\pm$ 0.8)	344,602 $\pm$ 38,493 (11.1 $\pm$ 1.1)
Total ^{14}C uptake	1,676,194 $\pm$ 111,993	3,091,085 $\pm$ 160,345
Total ^{14}C recovered	1,575,110 $\pm$ 87,466 (94.2 $\pm$ 1.9)	2,831,163 $\pm$ 121,091 (91.8 $\pm$ 1.5)

¹ Denotes fractions and sub-fractions plotted in Figure 5.5.

replicates; Figure 5.4b). The linearity of ^{14}C uptake and redistribution by WT cultures led to the transgenic lines (which have similar growth rates to the WT) being fed with $[\text{U-}^{14}\text{C}]\text{glucose}$ for 24 h before calculating biomass output.

Redistribution of ^{14}C in *ATCS* and control BY-2 lines

Biomass composition was determined after 24 h incubation with $[\text{U-}^{14}\text{C}]\text{glucose}$ as described for measuring linearity of ^{14}C uptake and redistribution. No transgenic culture was estimated to have metabolised more than 38% of the $[\text{U-}^{14}\text{C}]\text{glucose}$ supplied, and there were no significant differences in ^{14}C uptake between control and *ATCS* lines (Appendix 5.5). Given that the WT consumed a greater amount (43%) of supplied $[\text{U-}^{14}\text{C}]\text{glucose}$ linearly over 24 h, there was judged to have been sufficient glucose remaining at the end of the experiment to conclude that uptake of $[\text{U-}^{14}\text{C}]\text{glucose}$ by the transgenic lines had also been linear over the duration of the experiment.

ATCS line 6 was no different to the two control lines with respect to metabolite fractions (Figure 5.5). *ATCS* line 16 had approximately twice as much starch as and 20% more cell wall than the two control lines ($p < 0.05$, student's t test comparisons with each line). Starch content was 2.4 ± 0.2 times that of *ATCS* line 6 ($p < 0.005$, significant after Bonferroni correction for multiple comparisons). ^{14}C content of *ATCS* line 16 cell wall was not significantly different to that of *ATCS* line 6 when Bonferroni correcting for multiple comparisons, although the observed difference is similar to the differences between *ATCS* 16 and the control lines, and therefore seems likely to represent a real difference. 36-40% of ^{14}C was captured as CO_2 from each line. This is consistent with the $38\% \pm 2\%$ of ^{14}C redistributed into CO_2 by the WT (Appendix 5.6) and suggests a carbon conversion efficiency of between 60% and 64% by each line.

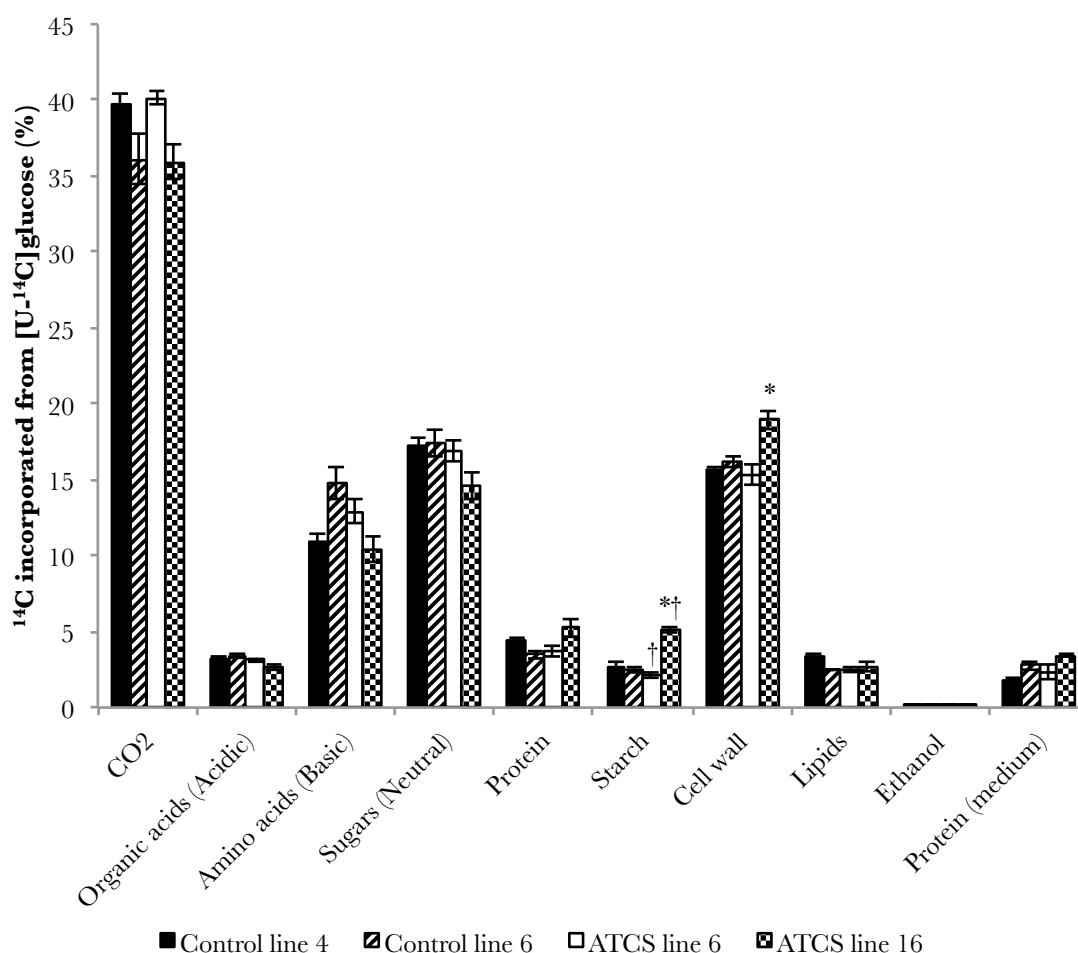


Figure 5.5. Redistribution of ^{14}C following feeding of cultures at metabolic steady state with $[\text{U-}^{14}\text{C}]\text{glucose}$.

Amino acid, sugar and organic acid fractions were obtained by ion-exchange chromatography of methanol-soluble metabolites. GC-MS analysis of unlabelled cultures grown in parallel indicated that these fractions were not completely pure. Error bars represent SEM, $n = 4$. “*” denotes significant differences of that line and each control line ($p < 0.05$). “†” denotes significant differences between *ATCS* lines 6 and 16 ($p < 0.005$, significant after Bonferroni-correction for multiple replicates). Recovery in the dataset was $98.35\% \pm 0.89\%$. There were no significant differences in recovery between lines. Values are compared with WT measurements in Appendix 5.6.

Determination of biomass output fluxes in wild-type, *ATCS* and control BY-2 lines

Cultures grown with unlabelled glucose were processed in parallel to radiolabelled cultures, and extracts were analysed by GC-MS using standard curves presented in Appendix 5.7. Combining this GC-MS data with the data on ^{14}C redistribution enabled the determination of output fluxes. These allow the feasible solution space of the MFA models to be constrained and so increase the determinability of the fluxes.

GC-MS data was corrected based on the turnover of metabolites calculated from isotopic steady state data. This was to remove pre-existing pools of metabolites from estimations of biomass output fluxes. Such pools are evidenced by ^{13}C enrichment at isotopic steady state not reaching the enrichment of the culture medium. Correction was performed by comparing the measured ^{13}C enrichment at isotopic steady state to the expected ^{13}C enrichment as described by Masakapalli (2011):

1. The concentration of a metabolite was divided by its relative molecular mass to derive its molar concentration.
2. The molar concentration was multiplied by the number of carbon atoms in each molecule to get the molar carbon concentration of the metabolite.
3. Multiplying the molar carbon concentration by the proportion of newly synthesised metabolite since sub-culturing (calculated by dividing the ^{13}C fractional enrichment of the metabolite at isotopic steady state by the expected fractional enrichment) provided the molar concentration of carbon being directed into the metabolite from the glucose supplied at the start of the experiment.
4. This value was then divided by the sum of the molar carbon concentrations of all the metabolites in the metabolic fraction to determine the proportion of carbon in each metabolite of that fraction.

5. Multiplying the proportion of carbon in each metabolite of a fraction by the ^{14}C content (disintegrations per minute) of that fraction then gave an estimate of the ^{14}C content of each of the metabolites in the fraction.
6. The ^{14}C content of each metabolite was expressed as an output flux relative to a glucose uptake of 10 by dividing the ^{14}C content of the metabolite by the number of carbons originating from glucose in the metabolite and multiplying this by 6 (the number of carbons in glucose).

Output flux estimates are presented in Table 5.2.

5.2.4. Implementation of steady-state metabolic flux analysis of wild-type, *ATCS* and transgenic control BY-2 cell suspension cultures

BY-2 cells were cultured in medium containing either $[1-^{13}\text{C}]$ glucose (99 mol% ^{13}C isotopic abundance), 20 mol% $[^{13}\text{C}_6]$ glucose or unlabelled glucose (control; ^{13}C at natural abundance) until isotopic and metabolic steady state was achieved. Three biological replicates of each feeding strategy were performed per genotype. Metabolites were extracted from harvested cultures and GC-MS was used to determine mass isotopomer information. Mass isotopomers define isotopomers with different masses. They do not individually provide information on isotopic arrangement, although information on isotopic arrangement can be derived by GC-MS due to the different fragments that arise during ionisation.

Table 5.2. Biomass output measurements supplied to 13C-FLUX models for wild-type, *ATCS* and control line BY-2 cell suspension cultures.

Values are expressed as net fluxes relative to glucose uptake of 10. Vupt1 and VUuptU differ from 10 and 2, respectively, due to carry-over of unlabelled glucose during sub-culture. These values were calculated from mass isotopomer measurements of glucose in cell culture filtrate. Vupt1 and VUuptU were used mutually exclusively in parallel models. For simplicity, sterol was defined in the network as a 5-carbon unit (derived from a single unit of isopentenyl diphosphate). Thus, rather than being divided by the carbon-content of each sterol and multiplied by six, each sterol was divided by 5/6 to get the output flux relative to glucose uptake. Sterol biomass was calculated using unlabelled hexane extracts. Sterol content as a proportion of dry weight (w/w) was used as a proxy for relative carbon content. Values are as supplied to the models. They are means $\pm$ standard deviations and are expressed either to two significant figures or to a maximum of four decimal places. Values (means and deviations) less than 0.0001 (underlined) were rounded up to 0.0001 in order to allow the model to direct some flux through those reactions.

Metabolite	Flux name	WT	Control line 4	Control line 6	<i>ATCS</i> line 6	<i>ATCS</i> line 16
[1- ¹³ C]glucose uptake	Vupt1	10 $\pm$ 0.18	9.6 $\pm$ 0.11	9.9 $\pm$ 0.21	9.4 $\pm$ 0.20	10 $\pm$ 0.22
[¹³ C ₆]glucose uptake	VUuptU	2.0 $\pm$ 0.037	1.9 $\pm$ 0.022	2.0 $\pm$ 0.041	1.9 $\pm$ 0.040	2.0 $\pm$ 0.044
Cell wall - hexosyl unit	Vwall	0.069 $\pm$ 0.0096	0.059 $\pm$ 0.0059	0.041 $\pm$ 0.0019	0.044 $\pm$ 0.010	0.063 $\pm$ 0.0031
Cell wall - pentosyl unit	Vpentaneff	0.086 $\pm$ 0.0025	0.060 $\pm$ 0.0006	0.065 $\pm$ 0.0006	0.051 $\pm$ 0.0074	0.055 $\pm$ 0.0006
Ethanol	Vadheff	0.14 $\pm$ 0.031	0.049 $\pm$ 0.013	0.057 $\pm$ 0.021	0.056 $\pm$ 0.025	0.061 $\pm$ 0.036
Lipid - acyl unit	Vfas1	0.59 $\pm$ 0.030	1.0 $\pm$ 0.11	0.75 $\pm$ 0.022	0.75 $\pm$ 0.099	0.80 $\pm$ 0.17
Lipid - glycerol unit	Vfas2	0.18 $\pm$ 0.076	0.043 $\pm$ 0.022	0.033 $\pm$ 0.013	0.045 $\pm$ 0.021	0.023 $\pm$ 0.0055
Starch - glucosyl unit	Vsta	0.25 $\pm$ 0.019	0.27 $\pm$ 0.052	0.25 $\pm$ 0.035	0.21 $\pm$ 0.036	0.51 $\pm$ 0.039
Sucrose - fructosyl unit	Vfsuc	0.51 $\pm$ 0.061	0.77 $\pm$ 0.056	0.72 $\pm$ 0.061	0.82 $\pm$ 0.061	0.59 $\pm$ 0.070
Sucrose - glucosyl unit	Vgsuc	0.51 $\pm$ 0.061	0.77 $\pm$ 0.056	0.72 $\pm$ 0.061	0.82 $\pm$ 0.061	0.59 $\pm$ 0.070
Sterol	Vsterol	0.018 $\pm$ 0.0005	0.024 $\pm$ 0.0016	0.028 $\pm$ 0.0009	0.031 $\pm$ 0.0019	0.026 $\pm$ 0.0014
Citrate	VcitOUT	0.017 $\pm$ 0.012	0.081 $\pm$ 0.0081	0.12 $\pm$ 0.010	0.058 $\pm$ 0.0088	0.064 $\pm$ 0.040
Malate	VmalOUT	0.23 $\pm$ 0.069	0.25 $\pm$ 0.037	0.21 $\pm$ 0.0064	0.21 $\pm$ 0.020	0.17 $\pm$ 0.029
Succinate	VsuccOUT	0.014 $\pm$ 0.0007	0.0052 $\pm$ 0.0007	0.0039 $\pm$ 0.0009	0.0058 $\pm$ 0.0016	0.0060 $\pm$ 0.0023
Alanine	Valaeff	0.056 $\pm$ 0.0057	0.064 $\pm$ 0.022	0.073 $\pm$ 0.028	0.081 $\pm$ 0.013	0.053 $\pm$ 0.021
Arginine	Vargeff	0.0043 $\pm$ 0.0013	0.0049 $\pm$ 0.0008	0.0033 $\pm$ 0.0003	0.0034 $\pm$ 0.0006	0.0056 $\pm$ 0.0010
Aspartate/Asparagine	Vaspeff	0.25 $\pm$ 0.064	0.13 $\pm$ 0.036	0.13 $\pm$ 0.015	0.12 $\pm$ 0.036	0.12 $\pm$ 0.028

Metabolite	Flux name	WT	Control line 4	Control line 6	ATCS line 6	ATCS line 16
Cysteine	Vcyseff	0.0068 ± 0.0015	0.0048 ± 0.0011	0.0037 ± 0.0014	0.0045 ± 0.0016	0.0054 ± 0.0020
Glutamate/Glutamine/Proline	Vglueff	0.66 ± 0.10	0.47 ± 0.12	0.60 ± 0.11	0.52 ± 0.100	0.52 ± 0.17
Glycine	Vglyeff	0.059 ± 0.016	0.030 ± 0.0059	0.048 ± 0.018	0.049 ± 0.021	0.021 ± 0.0052
Histidine	Vhiseff	0.0071 ± 0.0038	0.0074 ± 0.0021	0.0049 ± 0.0020	0.0052 ± 0.0004	0.0095 ± 0.0024
Isoleucine	Vileeff	0.033 ± 0.0011	0.026 ± 0.0021	0.020 ± 0.0014	0.022 ± 0.0054	0.030 ± 0.0080
Leucine	Vleueff	0.068 ± 0.0045	0.056 ± 0.0077	0.045 ± 0.010	0.045 ± 0.017	0.066 ± 0.021
Lysine	Vlyseff	0.033 ± 0.0008	0.040 ± 0.0059	0.036 ± 0.0028	0.037 ± 0.0053	0.039 ± 0.013
Methionine	Vmeteff	0.013 ± 0.0005	0.0099 ± 0.0006	0.0068 ± 0.0007	0.0078 ± 0.0016	0.011 ± 0.0027
Phenylalanine/Tyrosine	Vphe_tyreff	0.060 ± 0.0031	0.057 ± 0.0076	0.050 ± 0.0057	0.049 ± 0.0093	0.058 ± 0.014
Threonine	Vthreff	0.038 ± 0.012	0.054 ± 0.0093	0.075 ± 0.019	0.15 ± 0.028	0.053 ± 0.016
Tryptophan - 2C unit	Vtrp2caeff	<u>0.0001 ± 0.0001</u>	0.0016 ± 0.0012	0.0026 ± 0.0009	0.0011 ± 0.0002	0.0007 ± 0.0005
Tryptophan - 2C unit	Vtrp2cbeff	<u>0.0001 ± 0.0001</u>	0.0016 ± 0.0012	0.0026 ± 0.0009	0.0011 ± 0.0002	0.0007 ± 0.0005
Tryptophan - 3C unit	Vtrp3c_sereff	0.10 ± 0.029	0.12 ± 0.022	0.15 ± 0.030	0.11 ± 0.018	0.12 ± 0.029
Tryptophan - 4C unit	Vtrp4c	<u>0.0001 ± 0.0001</u>	0.0016 ± 0.0012	0.0026 ± 0.0009	0.0011 ± 0.0002	0.0007 ± 0.0005
Valine	Vvaleff	0.060 ± 0.0030	0.058 ± 0.013	0.055 ± 0.011	0.048 ± 0.0085	0.061 ± 0.012
γ-aminobutyrate	VgabaOUT	<u>0.0001 ± 0.0001</u>	<u>0.0001 ± 0.0001</u>	<u>0.0001 ± 0.0001</u>	<u>0.0001 ± 0.0001</u>	<u>0.0001 ± 0.0001</u>
Not included in model flux measurements section:						
CO ₂ efflux	Vco2out	23 ± 2.9	24 ± 0.87	22 ± 2.0	24 ± 0.54	22 ± 1.3

Correction for unlabelled pools of metabolites

Mass isotopomer measurements of soluble amino acids, protein hydrolysate, organic acids (citrate, fumarate and malate), starch and cell wall were used in the model. Proxy reactions were included to account for unlabelled pools of arginine, glycine, histidine, methionine and serine, which have carbon contributions from exogenous (unlabelled) CO₂ or MTHF.

In addition to the correction for unlabelled pools of glycine, methionine, serine, histidine and arginine by the inclusion of proxy reactions, other protein-derived amino acids, and sugars derived from starch and cell wall were corrected for pre-existing unlabelled pools using a mathematical procedure based on the labelling of primary metabolite precursors that were at near isotopic steady state (Masakapalli et al., 2013). This is detailed in Appendix 5.8.

GC-MS data included in the models

Mass isotopomer data considered for the models included soluble amino acids (including GABA), organic acids (citrate, fumarate, isocitrate, malate and succinate), protein hydrolysate, starch (glucose), cell wall (arabinose, galactose, glucose, mannose and xylose), soluble fructose and free sterols. Unfortunately, of six free sterols that were investigated, only sitosterol-1TMS (M-0 and M-90), stigmasterol-1TMS (M-0 and M-90), campesterol-1TMS (M-0 and M-90), cholesterol-1TMS (M-0 and M-90) and fucosterol-1TMS (M-98 and M-355) had measured ¹³C fractional enrichment in unlabelled samples of 0.0113 ± 0.03 . These fragments had ¹³C fractional enrichment approaching 20 mol% in cultures at isotopic steady state (with the exceptions of cholesterol and stigmasterol) but contained all 28 or 29 carbons of each compound and were therefore too large for GC-MS-derived information to be informative of isotopomeric composition. Therefore sterol mass isotopomer data was not included in the models, although sterol measurements were used as biomass output flux measurements. Table 5.3 details how each metabolite fragment is defined in the 13C-FLUX models.

Table 5.3. Definition of metabolites in 13C-FLUX models.

Metabolites were identified in different fractions arising from different extraction techniques by GC-MS. Amino acids (including protein hydrolysate) and organic acids were derivatised with TBDMS. Sugars (including from starch and cell wall) were derivatised with MeOX and TMS. Tryptophan is lost during protein hydrolysis. Glutamine and asparagine are converted to glutamate and aspartate, respectively, during protein hydrolysis. Arginine, glycine, histidine, methionine and serine from protein hydrolysate were differentiated from amino acids due to the inclusion of proxy reactions to account for the contribution of unlabelled pools to these metabolites in protein hydrolysate.

Fraction	Metabolite, as identified by GC-MS	Metabolite as defined in model
Soluble amino acids	Alanine	ALA
	Arginine	ARG
	Asparagine	ASP
	Aspartate	ASP
	Cysteine	CYS
	γ -amino-butyrate	GABA
	Glutamate	GLU
	Glutamine	GLU
	Glycine	GLY
	Histidine	HIS
	Isoleucine	ILE
	Leucine	LEU
	Lysine	LYS
	Methionine	MET
	Phenylalanine	PHE_TYR
	Proline	GLU
	Serine	SER
	Threonine	THR
	Tryptophan	TRP
	Tyrosine	PHE_TYR
	Valine	VAL
Protein hydrolysate	Alanine	ALA
	Arginine	ARG _{eff}
	Aspartate	ASP
	Cysteine	CYS
	Glutamate	GLU
	Glycine	GLY _{eff}
	Histidine	HIS _{eff}
	Isoleucine	ILE
	Leucine	LEU
	Lysine	LYS
	Methionine	MET _{eff}
	Phenylalanine	PHE_TYR
	Proline	GLU
	Serine	SER _{eff}
	Threonine	THR
	Tyrosine	PHE_TYR

Fraction	Metabolite, as identified by GC-MS	Metabolite as defined in model
Organic acids	Valine	VAL
	Citrate	CIT
	Fumarate	FUM
	Isocitrate	ICIT
	Malate	OAA
	Succinate	SUCC
Soluble sugars	Fructose	FSUC
Cell wall	Arabinose	PENTAN
	Galactose	WALL
	Glucose	WALL
	Mannose	WALL
	Xylose	PENTAN
Starch	Glucose	STA

Metabolite fragments arising from GC-MS were validated against specific criteria before inclusion in the model:

- ^{13}C fractional enrichment from cultures fed unlabelled glucose had to be 0.0113 ± 0.03 .
- Deviations in normalised isotopomer abundance across the GC-MS peak within a sample were less than 0.05, as calculated by the mass correction tool (Wahl et al., 2004). This takes into account the increased effect of noise on low-abundance samples and omits noisy and uninformative measurements.
- ^{13}C fractional enrichment of the metabolite in 20 mol% $[^{13}\text{C}_6]$ glucose samples had to be the same as the fractional enrichment of glucose in the CCF ± 0.03 for soluble metabolites, or ± 0.05 for protein, starch and cell wall components prior to mathematical correction for pre-existing unlabelled metabolite pools.
- Every mass isotopomer abundance value plus its deviation had to be greater than 0 for each metabolite fragment, such that fragments with mass isotopomers determined to have abundance of less than 0, and are therefore underestimated, were ignored.

Deviations in isotopomer values were adjusted to the maximum of either the deviation value determined by mass correction, the ^{13}C fractional enrichment of that fragment in extracts from cultures fed unlabelled glucose, or a GC-MS detector error of 0.01. This was done in order to best reflect the different sources of error that can impact on the perception of GC-MS fragment abundances.

Validation of model structure

Published Arabidopsis models have included cytosolic and plastidial pools of glucose 6-phosphate, a feature that was justified by the different labelling patterns of the glucosyl moieties of sucrose and starch (derived from the cytosolic and plastidial glucose 6-phosphate

pools, respectively) (Williams et al., 2008; Masakapalli et al., 2010). By contrast, flux models from oilseed rape (*Brassica napus*) (Schwender et al., 2003) and soybean (*Glycine max*) (Allen et al., 2009) embryos had no such differences in labelling and so used a single glucose 6-phosphate pool.

It was recently discovered that the fructosyl moiety contributes partially to the M-90 fragment of sucrose (Koubaa et al., 2012), which was previously thought to represent only the glucosyl moiety. This finding means that comparisons of sucrose and starch GC-MS measurements are unreliable and should not be used to determine if glucose 6-phosphate pools should be separated in the model. Instead, to gain an insight into the partitioning of glucose 6-phosphate, the relative abundance of each mass isotopomer in glucose from hydrolysate of starch and cell wall (which report on plastidial and cytosolic glucose 6-phosphate labelling, respectively) from cells fed with 99 mol% [1-¹³C]glucose were compared after correction for unlabelled pools. Analysis by GC-MS yielded two glucose peaks eluting at approximately 33.4 and 33.9 min and corresponding to different stereoisomers of the glucose (1MEOX) (5TMS) derivative. Comparisons between starch and cell wall-derived glucose revealed that the mass isotopomer distribution of the M-409 fragment, representing carbons 1 and 2, differed between the two pools, as determined by two-way analysis of variance ($p < 0.001$) (Figure 5.6). The mass isotopomer distribution of M-250 fragments (representing carbons 3, 4, 5 and 6) differed only in the second elution peak between both lines ($p < 0.001$). However, later modelling suggested that only this second peak was reliable for cell wall measurements, due to overestimation of the M+0 mass isotopomers in the earlier peak. This second peak had an M-409 m+1/m+0 ratio that was higher in cell wall than starch in each elution peak, whereas the M-250 m+1/m+0 ratio was lower in cell wall than starch. This suggested that more shuffling of the ¹³C had occurred before plastidial starch synthesis than cytosolic cell wall synthesis and supported the inclusion of separate glucose 6-phosphate pools.

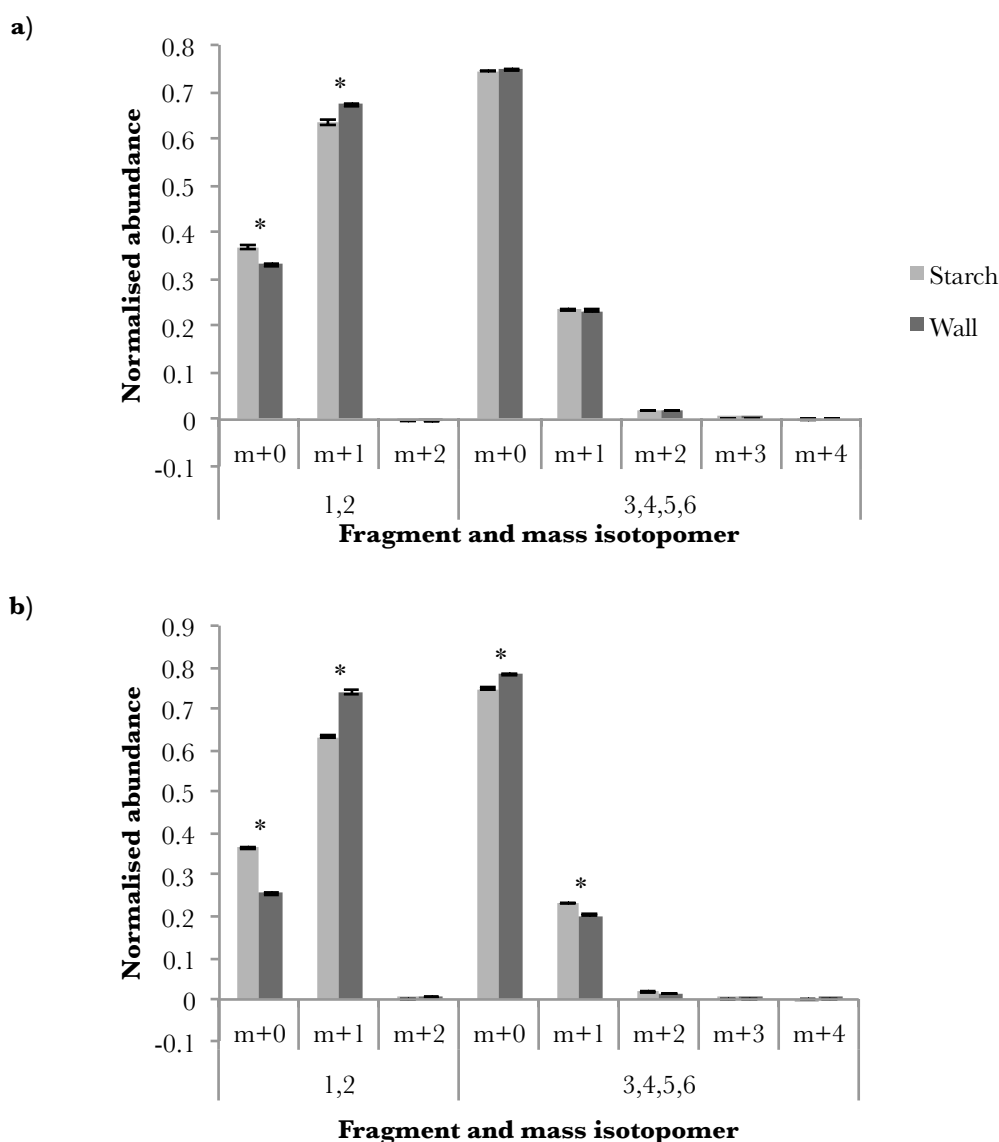


Figure 5.6. Mass isotopomer abundances of glucose (1MEOX) (5TMS) fragments from starch and wall extracted from wild-type BY-2 cell cultures fed [1-¹³C]glucose.

Mass isotopomers of glucose (1MEOX) (5TMS) from starch and cell wall were tested for similarity after mathematical correction for unlabelled pools. **a)** Early stereoisomer eluting at 33.4 min, **b)** Late stereoisomer eluting at 33.9 min. “*” denotes significant differences in normalised abundances between starch and cell wall ($p < 0.05$, student’s t tests). Error bars represent SEM of three replicates. Mass isotopomer abundance was normalised to the sum of the abundance of the different mass isotopomers reporting on that fragment of the metabolite.

Biomass output fluxes and ^{13}C -glucose uptake fluxes were initially set as free, but biomass data in Table 5.2 was included in the flux measurements section of each model. Models were run iteratively and the contribution of each isotopomer measurement to the residuum was investigated after each solution was obtained. Metabolite fragments were omitted from models if they contributed greatly to the residuum and if there was sound technical rationale for doing so. For instance, the majority of f302 fragments from amino acids had higher than expected abundance of the m+1 mass isotopomer. f302 fragments are often unreliable due to interference by the M-15 fragment, and so these fragments were usually omitted. In a related example, alanine M-15 had lower than expected abundance of the m+1 mass isotopomer in samples from $[1-^{13}\text{C}]$ glucose-fed cultures. This fragment was omitted because the M-57 fragment provided equivalent information with a better fit, and the M-15 fragment may be unreliable due to interference from the f302 fragment.

Biomass output net fluxes were constrained to the values determined during the modelling procedure after optimisation of the mass isotopomer dataset. These output fluxes were defined to two significant figures down to a minimum value of 0.0001. In most cases, the output fluxes predicted by the model were within the deviations of the values supplied in the flux measurements section. When model-predicted net fluxes were outside of these boundaries, the specific flux was constrained to the closest value within the measurement $\pm$ deviation range specified in the flux measurements section.

Establishment of best-fit models

Following omission of unreliable mass isotopomer data and constraint of biomass output fluxes, 1,000 MCSs with bootstrapping were performed on each model using the Donlp2 routine (P. Spelluci, Technische Universität Darmstadt, Germany). This provided flux analysis of simulated labelling measurements within bounds specified by the deviations supplied with the mass isotopomer data. Fluxes defining uptake of $[^{13}\text{C}]$ glucose and proxy reactions were

left free at this stage. This resulted in infeasible solutions that did not satisfy all the network constraints, feasible solutions with high residua, representing local minima, and feasible solutions with low residua.

Feasible results with low residua were analysed to assess the ability of the modelling procedure to determine the free fluxes and to create starting values for each free flux to be used in attaining global best-fit models of the original labelling data. The residuum cut-offs (Table 5.4) were chosen based on the distribution of residua (Figure 5.7) and principal component analysis of free flux values resulting from each simulation (Figure 5.8). Global best-fit models were determined by using the average free flux values of MCS with low residua as initial values in the modelling procedure.

5.2.5. Analysis of metabolic flux analysis results

Statistical analysis of metabolic flux models

All best-fit models passed χ^2 tests (Table 5.5) implying that they provide a reasonable explanation of the mass isotopomer data. The global best-fit model fluxes are presented in Table 5.6 and Appendix 5.9. The mass isotopomer measurements contributing to each model are presented in Appendix 5.10.

Linear statistical analysis of all net and exchange fluxes was performed using the EstimateStat component of 13C-FLUX. Results suggested that most fluxes of primary metabolism were well determined, with deviations less than the mean flux values. These findings were supported by non-linear analyses of free fluxes. These analyses took the form of calculating

Table 5.4. Monte Carlo simulation of mass isotopomer measurement during metabolic flux analysis.

1,000 Monte Carlo simulations of mass isotopomer data with bootstrapping were performed for each genotype. Histograms of residua distributions (Figure 5.10) and principal component analysis of free fluxes (Figure 5.11) were used to discern suitable residua cut-offs. Solutions with residua below the cut-offs were used to determine 68% confidence intervals for free fluxes and average free flux values were used as starting values when determining the global best fit solution for each line.

Genotype	Low/high residuum cut-off	Number of solutions		
		Below cut-off	Above cut-off	Infeasible
WT	3,800	277	64	659
Control line 4	4,200	287	300	413
Control line 6	4,400	256	46	698
ATCS line 6	4,500	347	83	570
ATCS line 16	4,500	216	279	505

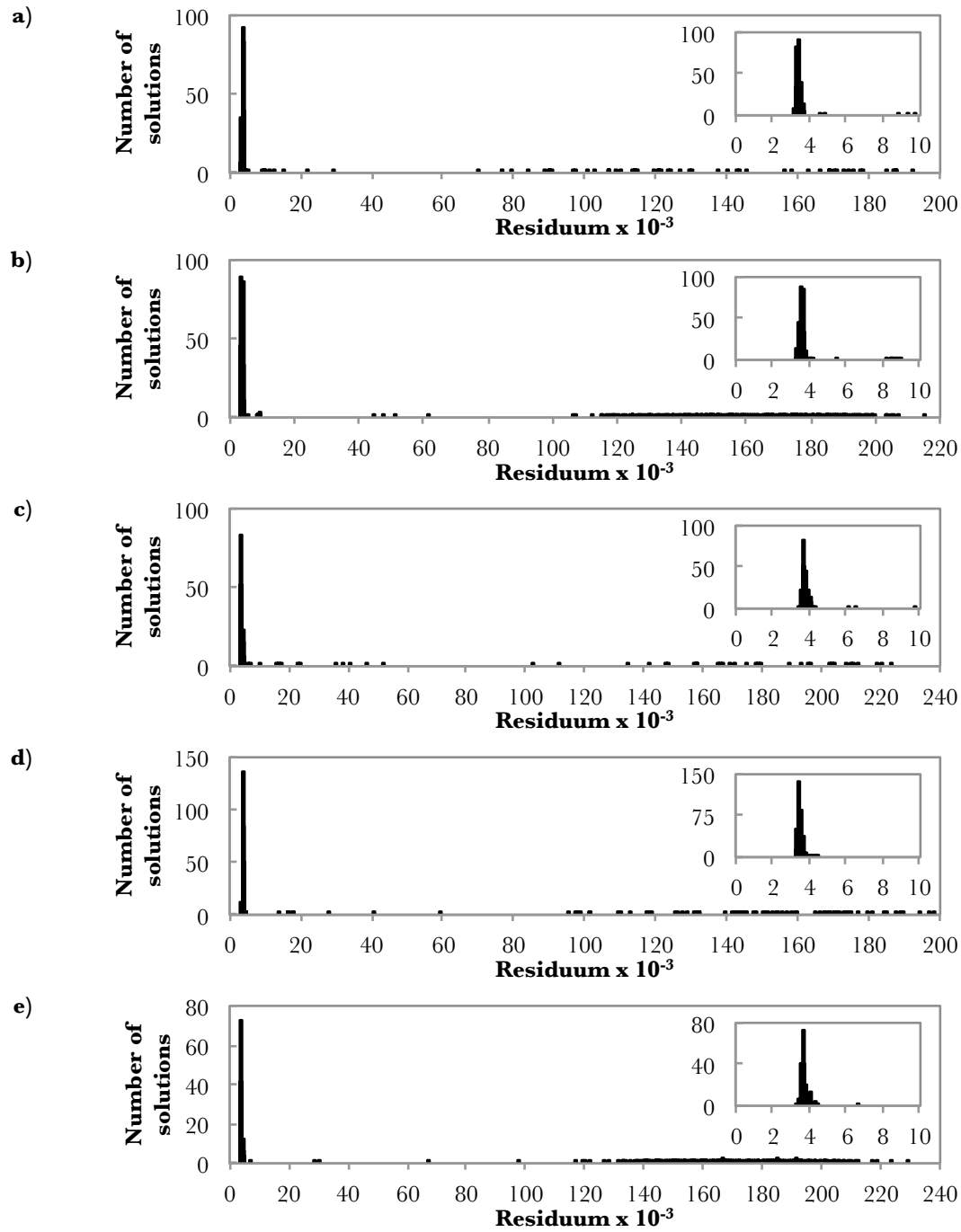


Figure 5.7. Histograms of residua from feasible Monte Carlo-simulated flux solutions.

Solutions were pooled into categories around each hundredth residuum. Insets provide further definition of distributions around the residuum cut-off. **a)** Wild-type (cut-off = 3,800), **b)** control line 4 (cut-off = 4,200), **c)** control line 6 (cut-off = 4,400), **d)** ATCS line 6 (cut-off = 4,500), **e)** ATCS line 16 (cut-off = 4,500).

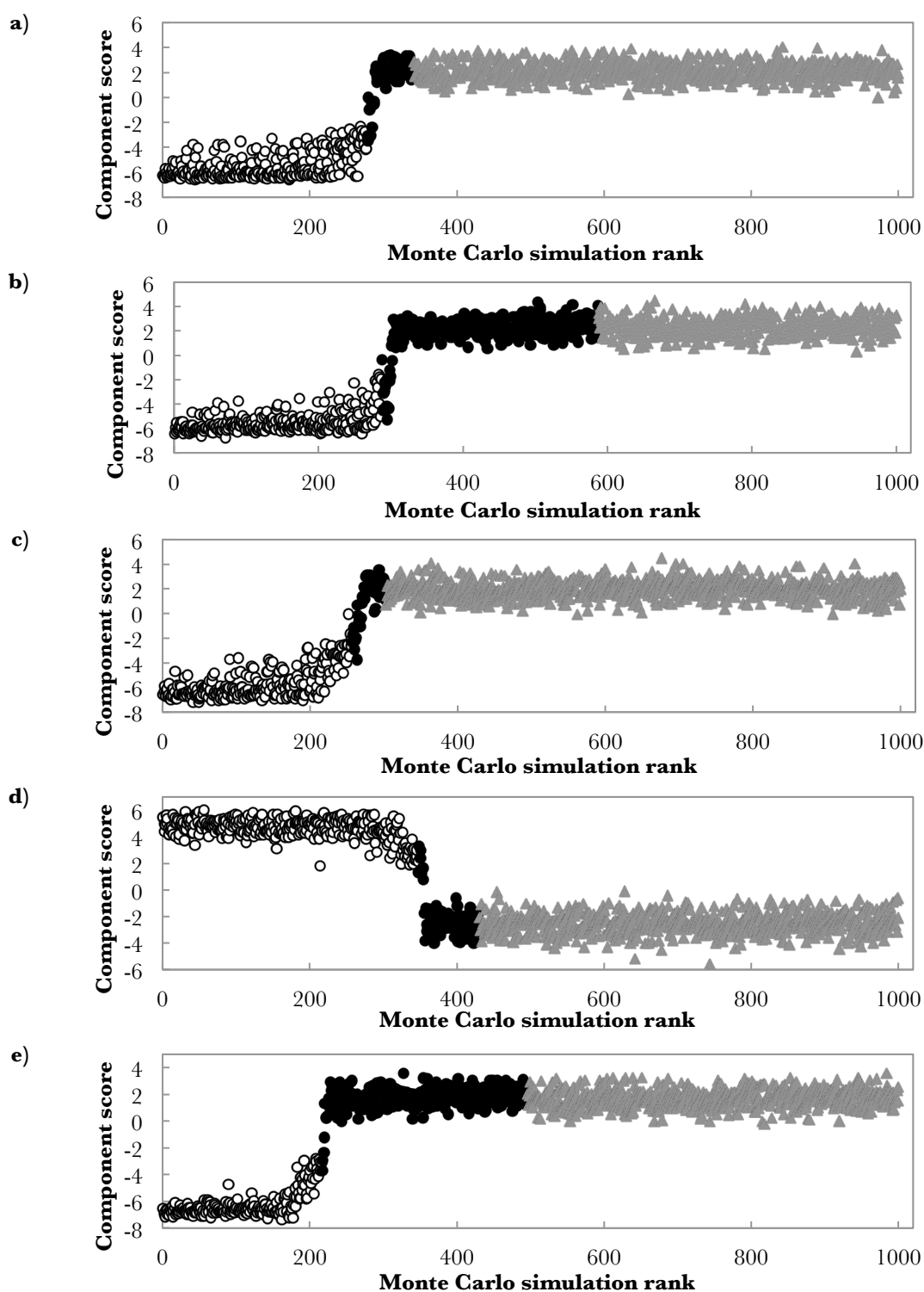


Figure 5.8. Principal component analysis of free flux values resulting from Monte Carlo-simulated mass isotopomer measurements.

Principal component one scores for MCS ranked by residuum after principal component analysis of unit-scaled free flux values. ○) Simulations with residua below cut-off, ●) feasible simulations with residua greater than cut-off, ▲) infeasible solutions. **a)** Wild-type, **b)** control line 4, **c)** control line 6, **d)** *ATCS* line 6, **e)** *ATCS* line 16.

Table 5.5. χ^2 tests of BY-2 steady state metabolic flux best-fit models.

All model passed χ^2 tests due to their residua being lower than the critical χ^2 value based on the number of degrees of freedom (isotopomer measurements minus free fluxes and metabolite fragments) and significance level (Q) of 0.95.

	WT	Control 4	Control 6	ATCS 6	ATCS 16
Free fluxes	35	35	35	35	35
Isotopomer measurements	3,054	3,056	3,451	3,106	3,100
Metabolite fragments	621	618	682	614	614
Degrees of freedom	2,398	2,403	2,734	2,457	2,451
Residuuum of global best-fit solution	907.1	1,085.3	915.2	949.4	1,126.5
Critical χ^2 value (Q = 0.95)	2,285.2	2,290.1	2,612.5	2,341.9	2,336.0
Model status (residuuum < critical χ^2)	Passed	Passed	Passed	Passed	Passed

Table 5.6. BY-2 steady state metabolic flux analysis results.

Fluxes are expressed as the best-fit flux $\pm$ the deviation derived by linear approximation using the EstimateStat command in 13C-FLUX. Fluxes for which no deviations are listed were constrained. Deviations marked as ND were not determinable by EstimateStat. 95% confidence intervals (upper and lower) are expressed in brackets. These were derived by non-linear statistical analysis of each free flux in turn using a confidence interval tool created for 13C-FLUX (Antoniewicz et al., 2006). Net flux lower confidence intervals marked as <0 were within the 95% confidence residuum range down to a flux of 0, but lower values were not testable due to constraints imposed by other fluxes or in the INEQUALITIES section of the model. Exchange fluxes that were within the 95% confidence residuum range with a flux of 0.99 were designated as having an upper confidence interval of infinity (∞). Comparisons of confidence intervals derived from Monte Carlo simulations with those from the confidence interval tool are provided in Appendix 5.6.

Italicised values indicate flux estimates with deviations greater than half of the best-fit value. Values in **bold** indicate *ATCS* fluxes that are significantly different to each of the control and WT measurements (z -score >1.961 , indicating that $>95\%$ of the assumed normal distribution lies between the two values). Underlined values indicate fluxes that differ significantly between the two *ATCS* lines. Fluxes that were constrained during the modelling process or are directly related to those (and so do not have deviations) were not considered. Differences in input and proxy fluxes reflect the amount of unlabelled glucose carried over during sub-culturing and the proximity to isotopic steady state. They should not be considered to reflect differences in metabolism between the lines.

	WT	Control line 4	Control line 6	<i>ATCS</i> line 6	<i>ATCS</i> line 16
Net fluxes					
Inputs					
Vupt1	10.000 $\pm$ 0.179	9.669 $\pm$ 0.179	10.000 $\pm$ 0.157	9.039 $\pm$ 0.205	9.477 $\pm$ 0.177
Vupt0	0.000 $\pm$ 0.179 (<0 , 0.103)	0.331 $\pm$ 0.179 (0.000, 0.630)	0.000 $\pm$ 0.157 (<0 , 0.205)	0.961 $\pm$ 0.205 (0.585, 1.208)	0.523 $\pm$ 0.177 (0.499, 1.054)
VUuptU	1.989 $\pm$ 0.006 (1.977, 2.002)	1.883 $\pm$ 0.006 (1.871, 1.895)	1.969 $\pm$ 0.006 (1.953, 1.978)	<u>1.877 $\pm$ 0.006 (1.864, 1.887)</u>	<u>1.927 $\pm$ 0.006 (1.910, 1.934)</u>
VUupt0	8.011 $\pm$ 0.006	8.117 $\pm$ 0.006	8.031 $\pm$ 0.006	<u>8.123 $\pm$ 0.006</u>	<u>8.073 $\pm$ 0.006</u>
Vco2in	<i>0.000 $\pm$ 0.882 (0.000, 1.639)</i>	<i>0.100 $\pm$ 0.969 (<0, 3.298)</i>	<i>1.656 $\pm$ 0.972 (0.331, 2.981)</i>	<i>0.000 $\pm$ 1.121 (<0, 0.819)</i>	<i>1.650 $\pm$ 0.941 (<0, 2.970)</i>
Vmthfin	3.316 $\pm$ 0.528 (2.735, 4.787)	3.229 $\pm$ 0.574 (2.617, 4.451)	2.098 $\pm$ 0.474 (1.694, 2.895)	2.434 $\pm$ 0.486 (0.730, 3.407)	1.310 $\pm$ 0.438 (0.783, 2.357)

	WT	Control line 4	Control line 6	ATCS line 6	ATCS line 16
Cytosolic hexose/triose phosphate metabolism					
Vg	10.000	10.000	10.000	10.000	10.000
Vgf	4.786 ± 1.120 (2.839, 6.731)	3.440 ± 2.204 (0.926, 5.953)	2.523 ± 1.872 (0.519, 3.023)	0.133 ± 2.962 (-0.634, 0.431)	4.944 ± 0.601 (2.967, 5.193)
Vald	4.266 ± 1.120	2.670 ± 2.204	1.793 ± 1.872	-0.687 ± 2.962	4.344 ± 0.601
Vtpi	4.086 ± 1.120	2.627 ± 2.204	1.760 ± 1.872	-0.732 ± 2.962	4.321 ± 0.601
Vfas2	0.180	0.043	0.033	0.045	0.023
Vpk	4.510 ± 0.356	3.778 ± 0.496	4.868 ± 0.419	<u>5.916 ± 0.668</u>	<u>4.160 ± 0.412</u>
Vpyr	3.912 ± 0.355	3.466 ± 0.458	4.207 ± 0.394	<u>5.549 ± 0.652</u>	<u>3.638 ± 0.402</u>
Vadh	0.140	0.049	0.058	0.055	0.062
Cytosolic PPP					
Vppp1	3.363 ± 2.915 (1.681, 4.036)	3.411 ± 4.740 (3.241, 6.141)	2.318 ± 3.113 (2.264, 4.180)	0.029 ± 1.686 (<0, 0.410)	0.654 ± 1.602 (0.577, 1.310)
Sugars storage					
Vgsuc	0.510	0.770	0.730	0.810	0.600
Vgsuceff	0.510	0.770	0.730	0.810	0.600
Vfsuc	0.520	0.770	0.730	0.820	0.600
Vfsuceff	0.520	0.770	0.730	0.820	0.600
Plastidial hexose/triose phosphate metabolism					
Vstsp	0.250	0.270	0.260	0.210	0.510
Vgfp	0.542 ± 1.229	1.990 ± 2.380	2.511 ± 1.972	<u>6.180 ± 2.987</u>	<u>0.160 ± 0.599</u>
Valdp	3.022 ± 1.081	4.239 ± 2.225	5.069 ± 1.877	7.873 ± 2.963	2.579 ± 0.560
Vpkp	0.457 ± 0.060 (0.343, 0.686)	1.021 ± 0.290 (0.511, 1.338)	0.452 ± 0.174 (0.000, 0.901)	0.645 ± 0.074 (0.486, 0.668)	0.539 ± 0.065 (0.404, 0.673)
Vpdhp	0.658	1.056	0.805	0.798	0.786
Vfas1	0.590	1.000	0.750	0.750	0.720
Vmep	0.122 ± 0.010	0.130 ± 0.015	0.080 ± 0.011	0.172 ± 0.011	0.162 ± 0.012
Plastidial PPP					
Vppp1_p	0.394 ± 3.741 (<0, 3.277)	0.000 ± 5.549 (<0, 2.670)	1.552 ± 3.752 (1.479, 1.714)	2.542 ± 1.764 (1.879, 2.607)	3.014 ± 1.921 (2.942, 5.422)
Vppp2a_p	2.480 ± 0.587	2.249 ± 0.595	2.557 ± 0.465	1.694 ± 0.296	2.419 ± 0.306
Vppp2b_p	1.210 ± 0.293	1.095 ± 0.297	1.252 ± 0.233	0.822 ± 0.148	1.179 ± 0.153
Vppp2c_p	1.270 ± 0.293	1.154 ± 0.297	1.305 ± 0.233	0.872 ± 0.148	1.239 ± 0.153
Vppp3a_p	1.270 ± 0.293	1.154 ± 0.297	1.305 ± 0.233	0.872 ± 0.148	1.239 ± 0.153

	WT	Control line 4	Control line 6	ATCS line 6	ATCS line 16
Vppp3b_p	1.270 ± 0.293	1.154 ± 0.297	1.305 ± 0.233	0.872 ± 0.148	1.239 ± 0.153
Cytosol-plastid exchange					
Vhcp	1.186 ± 3.374	2.260 ± 5.202	4.323 ± 3.674	8.932 ± 3.933	3.684 ± 1.813
Vtpt	1.952 ± 2.195	0.189 ± 4.318	-2.920 ± 3.743	-9.599 ± 5.602	2.426 ± 1.060
Vpyrt	0.401 ± 0.062 (0.201, 0.502)	0.199 ± 0.290 (<0, 0.545)	0.528 ± 0.175 (0.000, 1.051)	0.232 ± 0.079 (0.000, 0.349)	0.407 ± 0.068 (0.200, 0.601)
TCA cycle					
Vpdh	4.307 ± 0.374	3.584 ± 0.455	4.412 ± 0.400	6.396 ± 0.706	4.398 ± 0.480
Vcs	4.307 ± 0.375	3.584 ± 0.464	4.412 ± 0.403	6.396 ± 0.723	4.391 ± 0.468
Vca	4.235 ± 0.375	3.431 ± 0.464	4.208 ± 0.403	6.244 ± 0.723	4.245 ± 0.468
Vicdh	4.235 ± 0.375	3.431 ± 0.464	4.208 ± 0.403	6.244 ± 0.723	4.245 ± 0.468
Vakgdh	3.521 ± 0.375	2.946 ± 0.464	3.495 ± 0.403	5.620 ± 0.723	3.579 ± 0.468
Vsuccdh	3.507 ± 0.375	2.941 ± 0.464	3.491 ± 0.403	5.615 ± 0.723	3.573 ± 0.468
Vfum1	1.755 ± 0.188	1.473 ± 0.232	1.747 ± 0.201	2.809 ± 0.361	1.790 ± 0.234
Vfum2	1.755 ± 0.188	1.473 ± 0.232	1.747 ± 0.201	2.809 ± 0.361	1.790 ± 0.234
Vme	0.395 ± 0.114 (0.293, 0.587)	0.118 ± 0.090 (0.044, 0.416)	0.206 ± 0.101 (0.153, 0.498)	0.847 ± 0.166 (0.292, 1.236)	0.760 ± 0.161 (0.683, 1.222)
Vpepc	1.891 ± 0.101	1.329 ± 0.083	1.605 ± 0.098	2.264 ± 0.174	2.079 ± 0.137
Mevalonate isoprenoid biosynthesis pathway					
Vacl	0.054	0.072	0.084	0.093	0.078
Vmva1	0.018	0.024	0.028	0.031	0.026
Vmva2	0.018	0.024	0.028	0.031	0.026
Vsterol	0.018	0.024	0.028	0.031	0.026
TCA cycle efflux					
VcitOUT	0.018	0.081	0.120	0.059	0.068
VsuccOUT	0.014	0.005	0.004	0.006	0.006
VmalOUT	0.250	0.250	0.210	0.210	0.170
Vgabaeff	0.000	0.000	0.000	0.000	0.000
VgabaOUT	0.000	0.000	0.000	0.000	0.000
Efflux					
Vco2out	24.128 ± 1.235	22.342 ± 1.346	25.253 ± 1.313	26.835 ± 1.843	25.580 ± 1.459

	WT	Control line 4	Control line 6	ATCS line 6	ATCS line 16
Vwall	0.069	0.059	0.041	0.044	0.063
Vsta	0.250	0.270	0.260	0.210	0.510
Vadheff	0.140	0.049	0.058	0.055	0.062
Vpentan	0.086	0.060	0.065	0.051	0.055
Vpentaneff	0.086	0.060	0.065	0.051	0.055
Amino acid oxidation					
	0.000 ± 0.119 (-0.003, 0.205)	0.000 ± 0.099 (-0.003, 0.103)	0.000 ± 0.112 (-0.001, 0.213)	0.000 ± 0.132 (-0.007, 0.058)	-0.006 ± 0.139 (-0.019, 0.058)
Vthrox					
Amino acid metabolism					
Vglu	0.710	0.480	0.710	0.620	0.660
Vglueff	0.710	0.480	0.710	0.620	0.660
Vasp	0.349 ± 0.119	0.225 ± 0.099	0.239 ± 0.112	0.313 ± 0.132	0.214 ± 0.139
Vaspeff	0.260	0.130	0.130	0.130	0.120
Varg	0.004	0.005	0.003	0.003	0.006
Vargeff	0.004	0.005	0.003	0.003	0.006
Vasp_arg	0.004	0.005	0.003	0.003	0.006
Vser	8.628 ± 0.332	8.626 ± 0.402	7.918 ± 0.351	<u>6.226 ± 0.668</u>	<u>8.106 ± 0.456</u>
Vcys	0.007	0.005	0.004	0.005	0.005
Vcyseff	0.007	0.005	0.004	0.005	0.005
Vgly	8.521 ± 0.332	8.501 ± 0.402	7.764 ± 0.351	<u>6.112 ± 0.668</u>	<u>7.980 ± 0.456</u>
Vglydc	8.464 ± 0.356 (7.600, 9.289)	8.471 ± 0.416 (7.625, 8.896)	7.715 ± 0.365 (7.543, 8.124)	<u>6.064 ± 0.660 (4.318, 6.386)</u>	<u>7.953 ± 0.478 (7.789, 8.386)</u>
Vglyeff	0.058	0.030	0.049	0.048	0.021
Vmthfout	20.281 ± 0.917	20.184 ± 1.027	17.566 ± 0.861	14.597 ± 1.634	17.222 ± 1.068
Vala	0.056	0.064	0.076	0.081	0.054
Valaeff	0.056	0.064	0.076	0.081	0.054
Varo1	0.030	0.029	0.025	0.025	0.030
Varo2	0.030	0.029	0.025	0.025	0.030
Vleu1	0.128	0.114	0.099	0.096	0.126
Vleu	0.068	0.056	0.055	0.048	0.066
Vleueff	0.068	0.056	0.055	0.048	0.066
Vval	0.060	0.058	0.044	0.048	0.060

	WT	Control line 4	Control line 6	ATCS line 6	ATCS line 16
Vvaleff	0.060	0.058	0.044	0.048	0.060
Vmet	0.013	0.010	0.007	0.008	0.011
Vmeteff	0.013	0.010	0.007	0.008	0.011
Vthr	<i>0.072 ± 0.119</i>	<i>0.080 ± 0.099</i>	<i>0.099 ± 0.112</i>	<i>0.172 ± 0.132</i>	<i>0.078 ± 0.139</i>
Vthreff	0.039	0.054	0.079	0.150	0.054
Vile	0.033	0.026	0.020	0.022	0.030
Vileeff	0.033	0.026	0.020	0.022	0.030
Vphe_tyr	0.060	0.057	0.050	0.049	0.059
Vphe_tyreff	0.060	0.057	0.050	0.049	0.059
Vlys	0.019 ± 0.001	0.021 ± 0.002	0.021 ± 0.001	0.021 ± 0.001	0.018 ± 0.001
Vlys1	0.014 ± 0.001 (0.010, 0.017)	0.019 ± 0.002 (0.014, 0.024)	0.015 ± 0.001 (0.011, 0.019)	<u>0.016 ± 0.001 (0.012, 0.020)</u>	<u>0.021 ± 0.001 (0.016, 0.025)</u>
Vlyseff	0.033	0.040	0.036	0.037	0.039
Vhis	0.007	0.007	0.005	0.005	0.010
Vhiseff	0.007	0.007	0.005	0.005	0.010
Vtrp2ca	0.000	0.002	0.003	0.001	0.001
Vtrp2caeff	0.000	0.002	0.003	0.001	0.001
Vtrp2cb	0.000	0.002	0.003	0.001	0.001
Vtrp2cbef	0.000	0.002	0.003	0.001	0.001
Vtrp3c_sereff	0.100	0.120	0.150	0.110	0.120
Vtrp4c	0.000	0.002	0.003	0.001	0.001
Proxy reactions					
Vserin	0.005 ± 0.001 (0.003, 0.008)	0.003 ± 0.001 (0.000, 0.004)	0.004 ± 0.001 (0.000, 0.009)	<u>0.004 ± 0.001 (0.002, 0.007)</u>	<u>0.001 ± 0.001 (<0, 0.003)</u>
Vglyin	0.002 ± 0.001 (<0, 0.008)	0.001 ± 0.000 (<0, 0.004)	<i>0.001 ± 0.001 (<0, 0.004)</i>	<i>0.001 ± 0.001 (<0, 0.005)</i>	0.000 ± 0.000 (<0, 0.001)
	0.000 ± 0.000 (0.000, 0.000)	0.000 ± 0.000 (0.000, 0.001)	0.000 ± 0.000 (0.000, 0.000)	0.000 ± 0.000 (0.000, 0.000)	0.000 ± 0.000 (0.000, 0.000)
Vargin				0.000	
Vhisin	0.000 ± 0.000 (0.000, 0.001)	0.001 ± 0.000 (0.000, 0.001)	0.000 ± 0.000 (0.000, 0.001)	0.000 ± 0.000 (0.000, 0.001)	0.001 ± 0.000 (0.000, 0.001)
	0.001 ± 0.000 (0.001, 0.001)	0.001 ± 0.000 (0.001, 0.001)	0.001 ± 0.000 (0.001, 0.001)	0.001 ± 0.000 (0.001, 0.001)	0.001 ± 0.000 (0.001, 0.001)
Vmetin				0.001	
Vserprotout	0.105 ± 0.001	0.123 ± 0.001	0.154 ± 0.001	<u>0.114 ± 0.001</u>	<u>0.121 ± 0.001</u>
Vglyprotout	0.060 ± 0.001	0.031 ± 0.000	0.050 ± 0.001	<u>0.049 ± 0.001</u>	0.021 ± 0.000
Vargprotout	0.005 ± 0.000	0.005 ± 0.000	0.004 ± 0.000	0.004 ± 0.000	0.006 ± 0.000
Vhisprotout	0.008 ± 0.000	0.008 ± 0.000	0.005 ± 0.000	0.006 ± 0.000	0.010 ± 0.000

	WT	Control line 4	Control line 6	ATCS line 6	ATCS line 16
Vmetprotout	0.014 ± 0.000	0.011 ± 0.000	0.007 ± 0.000	<u>0.009 ± 0.000</u>	<u>0.012 ± 0.000</u>
Exchange fluxes					
Inputs					
Vupt1	0.000	0.000	0.000	0.000	0.000
Vupt0	0.000	0.000	0.000	0.000	0.000
VUuptU	0.000	0.000	0.000	0.000	0.000
VUupt0	0.000	0.000	0.000	0.000	0.000
Vco2in	0.000	0.000	0.000	0.000	0.000
Vmthfin	0.000	0.000	0.000	0.000	0.000
Cytosolic hexose/triose phosphate metabolism					
Vg	0.000	0.000	0.000	0.000	0.000
Vgf	0.99 ± 0.003 (0.987, ∞)	0.99 ± 0.002 (0.989, ∞)	0.99 ± 0.003 (0.989, ∞)	0.99 ± 0.006 (0.975, ∞)	0.99 ± 0.003 (0.988, ∞)
Vald	0.862 ± 0.007 (0.841, 0.873)	0.929 ± 0.004 (0.923, 0.933)	0.892 ± 0.005 (0.882, 0.898)	0.831 ± 0.127 (0.754, 0.835)	0.911 ± 0.005 (0.898, 0.920)
Vtpi	0.918 ± 0.018 (0.896, 0.936)	0.963 ± 0.007 (0.951, 0.976)	0.929 ± 0.016 (0.905, 0.936)	0.99 ± 0.053 (0.941, ∞)	0.904 ± 0.011 (0.880, 0.914)
Vfas2	0.000	0.000	0.000	0.000	0.000
Vpk	0.000	0.000	0.000	0.000	0.000
Vpyr	0.000	0.000	0.000	0.000	0.000
Vadh	0.000	0.000	0.000	0.000	0.000
Cytosolic PPP					
Vppp1	0.000	0.000	0.000	0.000	0.000
Sugar storage					
Vgsuc	0.000	0.000	0.000	0.000	0.000
Vgsuceff	0.000	0.000	0.000	0.000	0.000
Vfsuc	0.000	0.000	0.000	0.000	0.000
Vfsuceff	0.000	0.000	0.000	0.000	0.000
Plastidial hexose/triose-phosphate metabolism					
Vstsp	0.000	0.000	0.000	0.000	0.000
Vgfp	0.951 ± 0.040 (0.903, 0.968)	0.99 ± 0.024 (0.965, ∞)	0.961 ± 0.037 (0.913, 0.968)	<u>0.99 ± 0.006 (0.987, ∞)</u>	<u>0.948 ± 0.013 (0.925, 0.959)</u>
Valdp	0.000	0.000	0.000	0.000	0.000

	WT	Control line 4	Control line 6	ATCS line 6	ATCS line 16
Vpkp	0.000	0.000	0.000	0.000	0.000
Vpdhp	0.000	0.000	0.000	0.000	0.000
VfasI	0.000	0.000	0.000	0.000	0.000
Vmep	0.000	0.000	0.000	0.000	0.000
Plastidial PPP					
Vppp1_p	0.000	0.000	0.000	0.000	0.000
	0.941 ± 0.016 (0.894, 0.954)	0.941 ± 0.016 (0.894, 0.953)	0.933 ± 0.013 (0.910, 0.948)	0.857 ± 0.021 (0.785, 0.910)	0.909 ± 0.016 (0.882, 0.916)
Vppp2a_p					
Vppp2b_p	0.872 ± 0.055 (0.788, 0.898)	0.919 ± 0.036 (0.907, 0.928)	0.892 ± 0.043 (0.845, 0.901)	<u>0.922 ± 0.014 (0.905, 0.934)</u>	<u>0.971 ± 0.016 (0.922, 0.980)</u>
Vppp2c_p	0.852 ± 164714.000 (0.763, 0.874)	0.969 ± ND (0.775, 0.99)	0.897 ± 218643.000 (0.715, 0.917)	0.833 ± ND (0.764, ∞)	0.826 ± 356690.000 (0.484, 0.889)
Vppp3a_p	0.935 ± 176735.000 (0.840, 0.979)	0.859 ± ND (0.769, 0.881)	0.897 ± 218643.000 (0.715, 0.917)	0.827 ± ND (0.758, ∞)	0.793 ± 345242.000 (0.464, 0.853)
Vppp3b_p	0.253 ± 0.200 (0.023, 0.684)	0.546 ± 0.098 (0.434, 0.769)	0.145 ± 0.227 (0.000, 0.378)	0.948 ± 0.013 (0.933, 0.966)	0.867 ± 0.029 (0.792, 0.924)
Cytosol-plastid exchange					
Vhcp	0.931 ± 0.006 (0.919, 0.938)	0.959 ± 0.005 (0.947, 0.967)	0.933 ± 0.008 (0.910, 0.947)	0.966 ± 0.007 (0.960, 0.972)	0.951 ± 0.005 (0.945, 0.961)
Vtpt	0.928 ± 0.019 (0.836, 0.959)	0.969 ± 0.011 (0.944, 0.99)	0.969 ± 0.021 (0.956, 0.991)	0.794 ± 0.420 (0.000, 0.99)	0.911 ± 0.020 (0.903, 0.951)
Vpyrt	0.000	0.000	0.000	0.000	0.000
TCA cycle					
Vpdh	0.000	0.000	0.000	0.000	0.000
Vcs	0.000	0.000	0.000	0.000	0.000
Vca	0.000	0.000	0.000	0.000	0.000
Vicdh	0.000	0.000	0.000	0.000	0.000
Vakgdh	0.000	0.000	0.000	0.000	0.000
Vsuccdh	0.000	0.000	0.000	0.000	0.000
Vfum1	0.972 ± 0.013 (0.947, ∞)	0.963 ± 0.019 (0.915, ∞)	0.988 ± 0.015 (0.964, ∞)	0.980 ± 0.012 (0.960, ∞)	0.99 ± 0.013 (0.965, ∞)
Vfum2	0.972 ± 0.013	0.963 ± 0.019	0.988 ± 0.015	0.980 ± 0.012	0.99 ± 0.013
Vme	0.000	0.000	0.000	0.000	0.000
Vpepc	0.000 ± 0.201 (0.000, 0.205)	0.000 ± 0.219 (0.000, 0.205)	0.000 ± 0.197 (0.000, 0.103)	0.074 ± 0.223 (<0, 0.221)	0.000 ± 0.235 (0.000, 0.103)

WT		Control line 4	Control line 6	ATCS line 6	ATCS line 16
Cytosolic mevalonate pathway					
Vacl	0.000	0.000	0.000	0.000	0.000
Vmva1	0.000	0.000	0.000	0.000	0.000
Vmva2	0.000	0.000	0.000	0.000	0.000
Vsterol	0.000	0.000	0.000	0.000	0.000
TCA cycle efflux					
VcitOUT	0.000	0.000	0.000	0.000	0.000
VsuccOUT	0.000	0.000	0.000	0.000	0.000
VmalOUT	0.000	0.000	0.000	0.000	0.000
Vgabaeff	0.000	0.000	0.000	0.000	0.000
VgabaOUT	0.000	0.000	0.000	0.000	0.000
Efflux					
Vco2out	0.000	0.000	0.000	0.000	0.000
Vwall	0.000	0.000	0.000	0.000	0.000
Vsta	0.000	0.000	0.000	0.000	0.000
Vadheff	0.000	0.000	0.000	0.000	0.000
Vpentan	0.000	0.000	0.000	0.000	0.000
Vpentaneff	0.000	0.000	0.000	0.000	0.000
Amino acid oxidation					
Vthrox	$0.000 \pm 0.002 (<0, 0.003)$	$0.000 \pm 0.004 (<0, 0.003)$	$0.000 \pm 0.002 (<0, 0.002)$	$0.000 \pm 0.003 (<0, 0.007)$	$0.000 \pm 0.150 (<0, 0.026)$
Amino acid metabolism					
Vglu	0.482 ± 14374700.000 $(0.000, \infty)$	$0.533 \pm 7315230.000 (0.000,$ $\infty)$	0.513 ± 13705400.000 $(0.000, \infty)$	0.512 ± 10753600.000 $(0.000, \infty)$	0.512 ± 15946100.000 $(0.000, \infty)$
Vglueff	0.000	0.000	0.000	0.000	0.000
Vasp	0.000	0.000	0.000	0.000	0.000
Vaspeff	0.000	0.000	0.000	0.000	0.000
Varg	0.000	0.000	0.000	0.000	0.000
Vargeff	0.000	0.000	0.000	0.000	0.000
Vasp_arg	0.000	0.000	0.000	0.000	0.000
Vser	0.000	0.000	0.000	0.000	0.000

	WT	Control line 4	Control line 6	ATCS line 6	ATCS line 16
Vcys	0.000	0.000	0.000	0.000	0.000
Vcyseff	0.000	0.000	0.000	0.000	0.000
Vgly	0.874 ± 0.005 (0.852, 0.895)	0.898 ± 0.005 (0.887, 0.904)	0.858 ± 0.006 (0.837, 0.869)	0.826 ± 0.016 (0.816, 0.837)	0.880 ± 0.007 (0.871, 0.893)
Vglydc	0.716 ± 0.018 (0.644, 0.788)	0.722 ± 0.019 (0.686, 0.758)	0.655 ± 0.025 (0.588, 0.685)	0.769 ± 0.023 (0.750, 0.789)	0.779 ± 0.017 (0.738, 0.796)
Vglyeff	0.000	0.000	0.000	0.000	0.000
Vmthfout	0.000	0.000	0.000	0.000	0.000
Vala	0.000	0.000	0.000	0.000	0.000
Valaeff	0.000	0.000	0.000	0.000	0.000
Varo1	0.000	0.000	0.000	0.000	0.000
Varo2	0.000	0.000	0.000	0.000	0.000
Vleu1	0.000	0.000	0.000	0.000	0.000
Vleu	0.000	0.000	0.000	0.000	0.000
Vleueff	0.000	0.000	0.000	0.000	0.000
Vval	0.000	0.000	0.000	0.000	0.000
Vvaleff	0.000	0.000	0.000	0.000	0.000
Vmet	0.000	0.000	0.000	0.000	0.000
Vmeteff	0.000	0.000	0.000	0.000	0.000
Vthr	0.000	0.000	0.000	0.000	0.000
Vthreff	0.000	0.000	0.000	0.000	0.000
Vile	0.000	0.000	0.000	0.000	0.000
Vileeff	0.000	0.000	0.000	0.000	0.000
Vphe_tyr	0.000	0.000	0.000	0.000	0.000
Vphe_tyreff	0.000	0.000	0.000	0.000	0.000
Vlys	0.000	0.000	0.000	0.000	0.000
Vlys1	0.000	0.000	0.000	0.000	0.000
Vlyseff	0.000	0.000	0.000	0.000	0.000
Vhis	0.000	0.000	0.000	0.000	0.000
Vhiseff	0.000	0.000	0.000	0.000	0.000
Vtrp2ca	0.000	0.000	0.000	0.000	0.000
Vtrp2caeff	0.000	0.000	0.000	0.000	0.000
Vtrp2cb	0.000	0.000	0.000	0.000	0.000
Vtrp2cbeff	0.000	0.000	0.000	0.000	0.000

	WT	Control line 4	Control line 6	<i>ATCS</i> line 6	<i>ATCS</i> line 16
Vtrp3c_sereff	0.000	0.000	0.000	0.000	0.000
Vtrp4c	0.000	0.000	0.000	0.000	0.000
Proxy reactions					
Vserin	0.000	0.000	0.000	0.000	0.000
Vglyin	0.000	0.000	0.000	0.000	0.000
Vargin	0.000	0.000	0.000	0.000	0.000
Vhisin	0.000	0.000	0.000	0.000	0.000
Vmetin	0.000	0.000	0.000	0.000	0.000
Vserprotout	0.000	0.000	0.000	0.000	0.000
Vglyprotout	0.000	0.000	0.000	0.000	0.000
Vargprotout	0.000	0.000	0.000	0.000	0.000
Vhisprotout	0.000	0.000	0.000	0.000	0.000
Vmetprotout	0.000	0.000	0.000	0.000	0.000

95% confidence intervals, by using a confidence interval tool to vary each free flux in turn and measure its effect on the best-fit residuum (Antoniewicz et al., 2006) (Table 5.6), and 68% confidence intervals, by taking the range of the middle 68% of values for each flux from MCS solutions with residua below the specified cut-offs (Appendix 5.9).

Validation of models by comparison of CO₂ efflux estimates

Comparisons were made between the model-estimated net CO₂ efflux ($V_{co2out} - V_{co2in}$) and the efflux determined by measurement of ¹⁴CO₂ produced by cultures incubated with [U-¹⁴C]glucose in section 5.2.3. V_{co2out} was a good predictor of ¹⁴CO₂ efflux in all models, and statistical analysis did not suggest that any differences between the two methods of deducing CO₂ efflux were significant (Figure 5.9). This was evidence of internal consistency in the model and demonstrated that the biomass output fluxes calculated by the model (within the deviations provided in the “flux measurements” section) did not lead to an estimation of carbon conversion efficiency that was significantly different to that determined experimentally by redistribution of ¹⁴C.

Comparisons between cell lines

The WT model is presented in Figure 5.10. Models for transgenic lines are presented in Figure 5.11. The five models were broadly similar with most variation in best-fit fluxes being in the distribution of glycolysis (Figure 5.12). Glycolytic flux was predominantly cytosolic in WT. It appears to be more evenly split between the cytosol and plastid in control lines, although the fluxes were not well enough determined to conclude that they were significantly different to WT fluxes (Table 5.6). Glycolytic flux is almost completely plastidial in *ATCS* line 6, but the differences to control lines and the WT are not significant. *ATCS* line 16 more closely resembles the WT with respect to glycolytic fluxes, with significantly less plastidial glucose 6-phosphate isomerase flux (V_{gfp}) and flux of triose phosphate/ P_i and

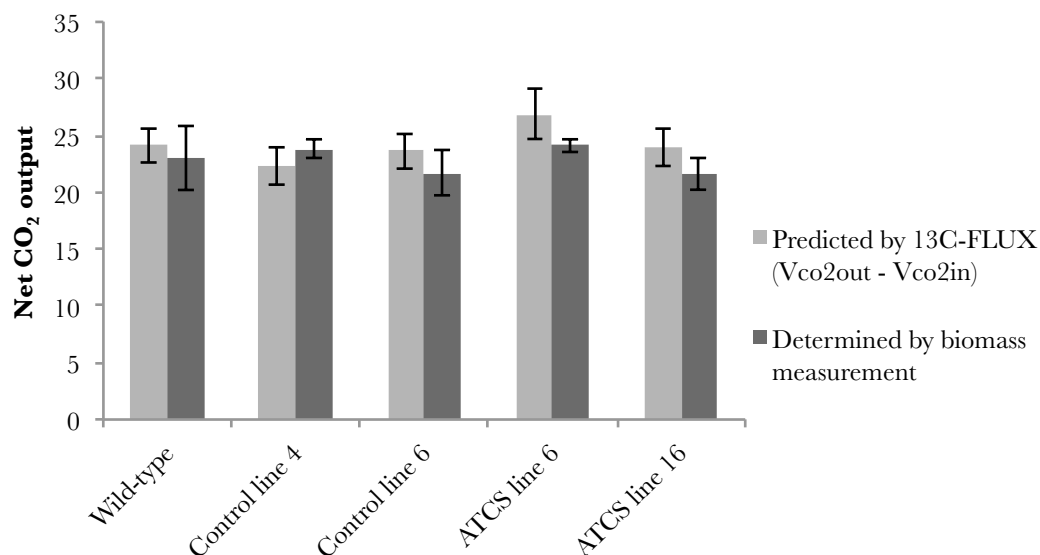


Figure 5.9. Model validation by comparison of CO₂ output estimates.

Net CO₂ output ($V_{co2out} - V_{co2in}$) estimated by 13C-FLUX was compared with CO₂ efflux determined from ¹⁴CO₂ measurements from cultures incubated with [U-¹⁴C]glucose. Model-estimated net fluxes are from the global best-fit models, with error bars representing deviations determined by EstimateStat. ¹⁴CO₂ measurements are from Table 5.2 and represent means of four biological replicates, with error bars indicating standard deviations. z -Scores were calculated to determine if the measurements were different in each line. This was done because the population size of 13C-FLUX estimation is unquantifiable. As such, student's t tests are inappropriate for comparing the measurements. z -scores were all <1.961, indicating that the means lie within 95% of a presumed normal distribution from each other. Differences were not deemed to be significant as a result.

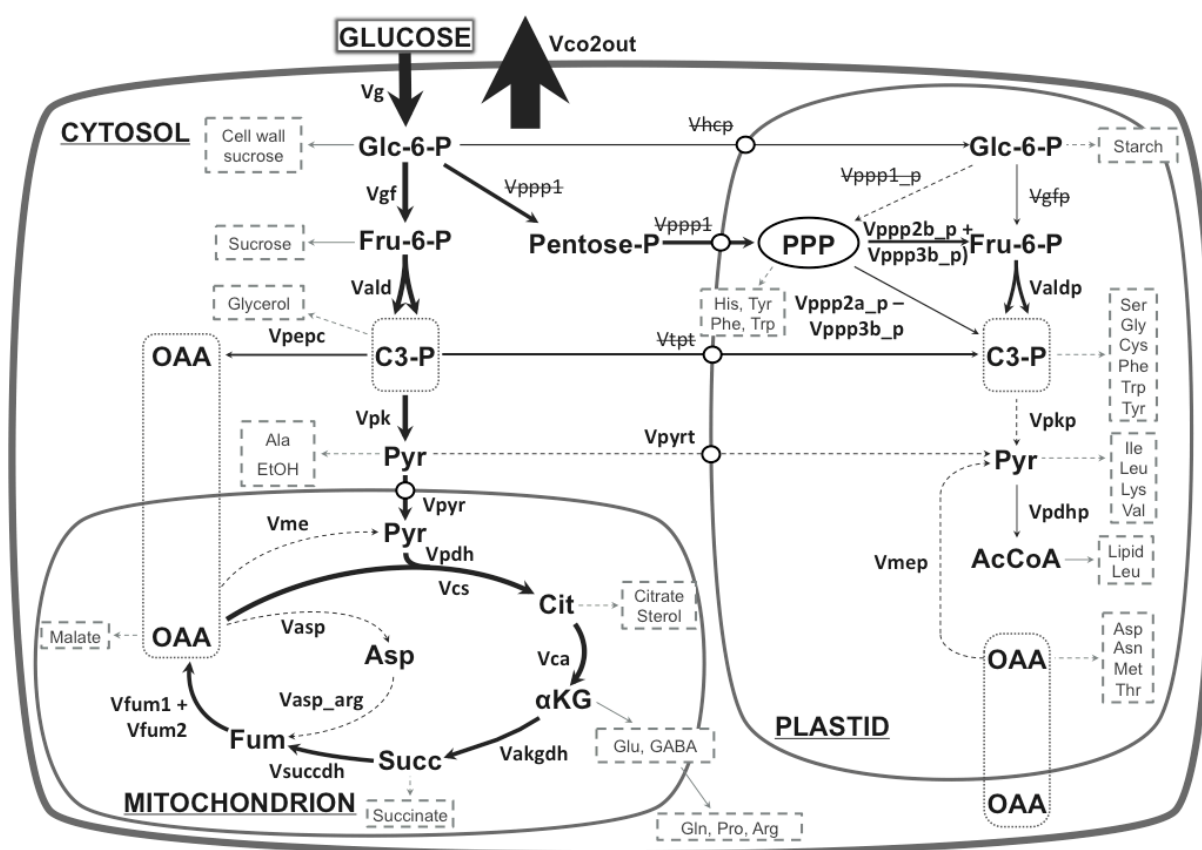


Figure 5.10. Schematic representation of wild-type BY-2 central metabolic fluxes.

Arrow thicknesses represent relative net fluxes from the best-fit flux maps. Dashed arrows indicate fluxes of less than 0.5. Poorly determined fluxes, with deviations greater than half their value, are struck through. Metabolites in dashed boxes indicate those that contribute to constraining the flux space through biomass output data or mass isotopomer measurements.

Abbreviations: Metabolites: **AcCoA**, acetyl-CoA; **αKG**, α-ketoglutarate; **Asp**, aspartate; **C3-P**, triosephosphate; **Cit**, citrate; **Fru-6-P**, fructose 6-phosphate; **Fum**, fumarate; **Glc-6-P**, glucose 6-phosphate; **OAA**, oxaloacetate; **Pyr**, pyruvate; **Pentose-P**, pentose phosphate; **PPP**, pentose phosphate pathway. Fluxes: **Vakgdh**, αKG dehydrogenase/succinyl-CoA synthetase; **Vald**, fru-6-P → C3-P/phosphoenolpyruvate; **Valdp**, plastidial fru-6-P → C3-P/phosphoenolpyruvate; **Vca**, aconitase/isocitrate dehydrogenase; **Vcs**, cit synthase; **Vfum1/-2**, fumarase; **Vg**, glucose uptake; **Vgf**, cytosolic glc-6-P isomerase; **Vgfp**, plastidial glc-6-P isomerase; **Vhcp**, plastidial glc-6-P/Pi translocator; **Vme**, mitochondrial malic enzyme; **Vmep**, plastidial malic enzyme; **Vpdh**, mitochondrial pyr dehydrogenase; **Vpdhp**, plastidial pyr dehydrogenase; **Vpepc**, phosphoenolpyruvate carboxylase; **Vpk**, pyr kinase; **Vpkp**, plastidial pyr kinase; **Vppp1**, cytosolic pentose phosphate pathway oxidative steps and plastidial uptake; **Vppp1_p**, plastidial pentose phosphate pathway oxidative steps; **Vppp2a_p/-2b_p/-3b_p**, plastidial pentose phosphate pathway non-oxidative steps; **Vpyr**, mitochondrial pyr uptake; **Vpyrt**, plastidial pyr uptake; **Vsucdh**, succ dehydrogenase; **Vtpt**, plastidial C3-P/Pi and phosphoenolpyruvate/Pi translocator.

phosphoenolpyruvate/ P_i from the plastid (Vtpt) than *ATCS* line 6 (Table 5.6; z -score >1.961 , indicating that $>95\%$ of the presumed normal distribution lies between the two values).

There was a high degree of similarity in the biomass derived from TCA cycle intermediates, but despite this *ATCS* line 6 TCA cycle net fluxes were considerably higher than in the other lines. Net Vcs was 1.4-1.8 times that of each of the other lines (Figure 5.13; z -score > 1.961). Other fluxes of the TCA cycle were correspondingly increased in *ATCS* line 6 (Table 5.6). *ATCS* line 16, by contrast, had TCA cycle fluxes approximately equal to the WT and control lines.

5.2.6. Comparisons of coenzyme demand and supply between BY-2 cell lines

Coenzyme demand was determined from the calculations of biomass output in section 5.2.3. Requirements of adenosine triphosphate (ATP), reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) for each biomass component were primarily taken from published data on biomass outputs for an heterotrophic *Arabidopsis* cell culture (Masakapalli et al., 2010). In addition to the metabolites quantified in that work, sterol was also measured. Reaction stoichiometries are listed in Appendix 5.11. This ignored the requirement for coenzymes in metabolite transport, futile metabolite cycles and DNA synthesis. There were no significant differences in coenzyme demand between either of the *ATCS* lines and either control line (Table 5.7a). The WT had a 21% greater demand for ATP per unit glucose uptake than the control lines and *ATCS* line 6 ($p < 0.05$, student's t tests). This is due to slightly increased levels of protein and amino acids being detected in the WT ($p < 0.05$, student's t tests, relative to each transgenic line; Appendix

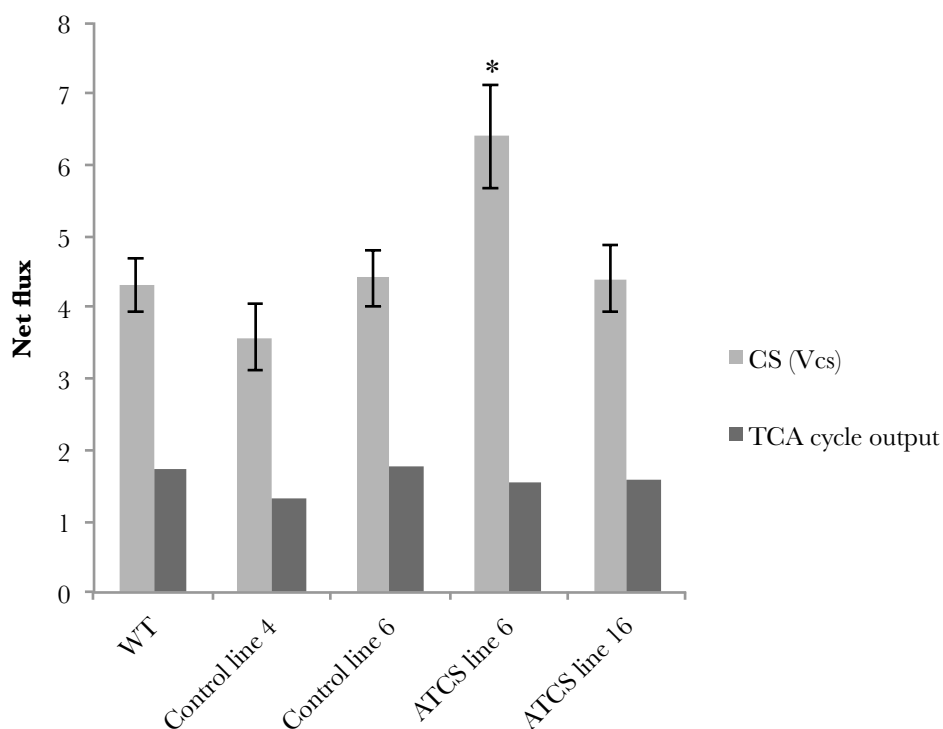


Figure 5.13. BY-2 cell line net citrate synthase fluxes and TCA cycle output fluxes.

Error bars on net flux through CS (Vcs) are deviations from EstimateStat. TCA cycle outputs are the sum of Varg, Vaspeff, VcitOUT, Vgabaeff, Vglu, Vlys, Vlys1, VmalOUT, Vmet, Vmva1, VsuccOUT and Vthr net fluxes. These fluxes result from the biomass measurements in Table 5.2. *ATCS* line 6 Vcs was significantly different to each of the other lines (z -score > 1.961).

Table 5.7. Coenzyme demand and supply.

Coenzyme demand was calculated using the biomass output data in Table 5.2. Equal assimilation of NH_4^+ and NO_3^- was assumed. Values are means of four biological replicates $\pm$ SEM. Coenzyme supply was calculated using the best-fit flux values in Table 5.6. All values are relative to glucose uptake (Vg) net flux of 1. Further details of calculations are in Appendix 5.11. ATP demand by the WT was significantly different to demand by each control line and *ATCS* line 6 ($p < 0.05$, student's t test).

a) Demand

	ATP	NADH	NADPH
WT	0.70 $\pm$ 0.01	0.17 $\pm$ 0.01	0.67 $\pm$ 0.03
Control line 4	0.60 $\pm$ 0.00	0.17 $\pm$ 0.00	0.58 $\pm$ 0.02
Control line 6	0.56 $\pm$ 0.02	0.16 $\pm$ 0.00	0.59 $\pm$ 0.03
<i>ATCS</i> line 6	0.58 $\pm$ 0.03	0.16 $\pm$ 0.00	0.62 $\pm$ 0.03
<i>ATCS</i> line 16	0.65 $\pm$ 0.05	0.16 $\pm$ 0.01	0.60 $\pm$ 0.04

b) Supply

	ATP	NADH	NADPH
WT	-0.68 ^a /5.59 ^{b,c} /5.23 ^{b,d}	2.12 ^c /1.70 ^d	0.75 ^c /1.17 ^d
Control line 4	-0.71 ^a /4.80 ^{b,c} /4.51 ^{b,d}	1.88 ^c /1.53 ^d	0.68 ^c /1.03 ^d
Control line 6	-0.59 ^a /5.87 ^{b,c} /5.51 ^{b,d}	2.17 ^c /1.75 ^d	0.77 ^c /1.19 ^d
<i>ATCS</i> line 6	-0.30 ^a /9.14 ^{b,c} /8.61 ^{b,d}	3.12 ^c /2.50 ^d	0.51 ^c /1.14 ^d
<i>ATCS</i> line 16	-0.66 ^a /5.66 ^{b,c} /5.31 ^{b,d}	2.13 ^c /1.70 ^d	0.73 ^c /1.16 ^d

^a ATP generated by substrate-level phosphorylation

^b Total ATP generated by substrate-level phosphorylation and oxidative phosphorylation, assuming that all NADH not contributing to biomass is used for ATP generation and that reduced flavin adenine dinucleotide (FADH₂) generation is 60% of NADH generation. Conservative stoichiometries of 2.3 ATP per NADH and 1.4 ATP per FADH₂ were assumed (Hinkle, 2005).

^c Coenzyme generation assuming all isocitrate dehydrogenase is NAD⁺-dependent.

^d Coenzyme generation assuming all isocitrate dehydrogenase is NADP⁺-dependent.

5.6), but could be an artefact incurred by assessing ^{14}C redistribution in the WT in a separate experiment to the analysis of the transgenic lines. This may have been due to differences in the efficiency of the ion exchange chromatography step of metabolite fractionation between the two experiments.

Coenzyme supply (Table 5.7b) was calculated from the best-fit flux values for reactions in primary metabolism (as described in Appendix 5.11). ATP production from primary metabolism (by substrate-level phosphorylation) was insufficient to meet demand in all lines. However, the NADH supply was at least 9 times greater than demand for biomass in each line. ATP demand for biosynthesis is easily met if excess NADH is assumed to be available for ATP synthesis by oxidative phosphorylation and if FADH_2 synthesis is assumed to be 60% of NADH synthesis. *ATCS* line 6 was estimated to be able to supply 1.4 and 1.6 times the levels of ATP or NADH, respectively, of control line 6, the next most productive line. This reflects the modelled increase in TCA cycle flux in this line. NADPH requirement for biomass could only be met in *ATCS* line 6 if at least 16% of isocitrate dehydrogenase (IDH) flux was NADP^+ -dependent. Other lines were capable of producing sufficient NADPH with only NAD^+ -dependent IDH flux due to the oxidative pentose phosphate pathway (*Vppp1* and *Vppp1_p*) carrying greater flux in these lines.

5.3. Discussion

5.3.1. Results summary

MFA of heterotrophic WT and transgenic *ATCS* and control BY-2 cell lines was performed using parallel $[1-^{13}\text{C}]$ glucose and 20 mol% $[^{13}\text{C}_6]$ glucose labelling experiments after

establishing isotopic steady state and determining biomass composition through the redistribution of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$. Models were validated by comparisons with $^{14}\text{CO}_2$ emitted in biomass determination experiments. The results did not reveal consistent differences in fluxes (including biosynthetic fluxes) between genotypes. However, TCA cycle flux in *ATCS* line 6 was determined to have increased to 1.5 times that of the other cell lines.

5.3.2. The chosen ^{13}C feeding strategy led to over-determination of tricarboxylic acid cycle fluxes but poor determination of parallel fluxes in the cytosol and plastid

Metabolic fluxes were generally well determined, with deviations of less than half the value of the best-fit flux estimate. The primary exceptions to this were net glycolytic and oxidative PPP fluxes, which were duplicated in the cytosol and plastid. These exceptions are indicative of difficulties in determining sugar phosphate partitioning, which is a common observation in MFA of plants (Lonien and Schwender, 2009) and is due to deficiencies in the labelling strategy; inclusion of an additional $[\text{2-}^{13}\text{C}]\text{glucose}$ labelling experiment and positional isotopomer data from NMR spectroscopy would probably have led to more precise flux estimates (Masakapalli et al., 2010).

GC-MS mass isotopomer data from $[\text{1-}^{13}\text{C}]\text{glucose}$ and $[\text{13C}_6]\text{glucose}$ provided good resolution of TCA cycle fluxes and revealed BY-2 cells to be a good model organism for plant metabolic flux analysis (MFA). It was TCA cycle fluxes in which particular interest lay, as it was hypothesised that increasing flux into citrate in the TCA cycle would facilitate an increase in the provision of cytosolic acetyl-CoA available for sterol biosynthesis. These results demonstrate the use of considering the different labelling and analytical strategies available for

MFA and the resolution that each will give to the specific area of metabolism in which interest lies. While other strategies may have given glycolytic and PPP fluxes with higher resolution, these fluxes were not deemed so relevant to the question of the significance of CS to TCA cycle flux control, and inclusion of additional strategies would have been significantly more time-consuming and costly.

5.3.3. Tobacco BY-2 cell culture metabolic fluxes were consistent with metabolic fluxes of other heterotrophic plant cell suspension cultures

The redistribution of ^{14}C into $^{14}\text{CO}_2$ indicates carbon conversion efficiency (CCE) in each BY-2 cell line of 60-64%. This is comparable to the 60-65% CCE observed for heterotrophic *Arabidopsis* cell suspension cultures grown in comparable conditions (Williams et al., 2008; Masakapalli et al., 2010; Masakapalli et al., 2013). Biomass composition was also similar to *Arabidopsis* cell cultures (Masakapalli et al., 2010; Masakapalli et al., 2013), with the exception of methanol-insoluble components. Of these, cell wall accounted for five times more biomass in BY-2 extracts, and there were corresponding decreases in starch and protein. The cell wall component was primarily indigestible cell wall, and so poor accessibility of α -amylase and amyloglucosidase to their substrates during the extraction could have resulted in an overestimation of cell wall at the expense of starch. The potential of unreliable starch and cell wall estimates is not thought likely to affect the quality of the flux analyses, as the cell wall and starch flux measurements fitted the labelling data well prior to being constrained, and this data provides information on the distribution of upstream glycolytic fluxes, which were generally poorly determined due to the labelling strategy.

Flux analyses of heterotrophic *Arabidopsis* suspension cells cultured in similar conditions have suggested that most glycolytic flux is plastidial (Williams et al., 2008; Masakapalli et al., 2013) or, alternatively, that the PPP is favoured over glycolysis, with most oxidative flux occurring in the cytosol (Masakapalli et al., 2010). Glycolytic flux appears to be favoured over the PPP in the BY-2 cultures. The determinability of the location of glycolytic flux varies by cell line, although when fluxes were well determined they tended to be split relatively evenly between the cytosol and plastid. In common with *Arabidopsis* suspension culture models, up to 10 times as much pyruvate kinase flux was cytosolic as plastidial, and only small amounts of flux involving oxaloacetate, acetyl-CoA and pyruvate occurred in the plastid.

As expected for a rapidly growing heterotrophic cell line (Sweetlove et al., 2010), the BY-2 models reveal a conventional cyclic TCA cycle flux in common with *Arabidopsis* (Williams et al., 2008; Masakapalli et al., 2010; Masakapalli et al., 2013) and tomato cell cultures (Rontein et al., 2002). However, TCA cycle fluxes were about 1.5 times as fast in *Arabidopsis* and twice as fast in tomato cells cultured under standard heterotrophic conditions. Given the similarities in CCE between BY-2 and *Arabidopsis* cell cultures, the difference in TCA cycle is surprising and the reasons for it are unclear.

Corresponding to the cyclic TCA flux mode in operation in BY-2 cells is an excess supply of NADH available for ATP biosynthesis. ATP demand for biosynthesis in BY-2 cells was 13% of the potential supply, similar to the situation in heterotrophic *Arabidopsis* cell cultures, where ATP demand for biosynthesis was only 15% of the potential ATP supply (Masakapalli et al., 2010). This suggests a high demand for ATP for transport processes and metabolite cycling reactions not included in the models by heterotrophic cell cultures.

5.3.4. Transgenic *ATCS* lines had different fluxomes but were not consistently different to control lines

There were very few differences in biomass composition between the *ATCS* and control transgenic lines, and no consistent differences between genotypes. Metabolomic results in Chapter 4.2.7.3 suggested that *ATCS* line 16 had greater methanol-soluble metabolite content than other lines when GC-MS peak areas were normalised to the peak area of a ribitol standard added prior to extraction. This was thought to be an indication of problems with the recovery of ribitol from *ATCS* line 16, and the analytical method was altered as a result. The data on the redistribution of ^{14}C from [U- ^{14}C]glucose did not reveal any significant differences in the yields of methanol-soluble metabolites between transgenic lines and corroborated the results in Chapter 4 achieved by normalising to alkane peak area rather than ribitol peak area.

There were also no consistent differences between genotypes in metabolic fluxes, but there were differences between transgenic lines. The MFA results suggest that *ATCS* line 6 had glycolytic flux primarily localised to the plastid, whereas other lines had glycolytic flux more evenly distributed between the cytosol and plastid. However, as already addressed, these fluxes were poorly determined making it unwise to draw conclusions on glycolysis localisation based on these results. Therefore the only statistically robust difference between this line and other lines was in TCA cycle flux, which was increased by at least 1.4 times and matches the prior expectation of increased CS activity.

ATCS line 16 had similar fluxes to control lines and the WT. This was despite the line having a greater proportion of insoluble metabolites (primarily starch and cell wall) than other lines. Thus, MFA does not hint at any obvious explanations for the altered biomass in this line.

5.3.5. Further investigation of *ATCS* line 6

Unlike the other BY-2 lines studied, the results suggest that *ATCS* line 6 was unable to meet demand for NADPH for biomass solely by the oxidative PPP. The requirement for flux through NADP⁺-dependent IDH in order for cultures to meet demand for NADPH has also been predicted in *Arabidopsis* cell cultures (Masakapalli et al., 2010; Masakapalli et al., 2013). This suggests that substantial IDH flux occurs outside of the conventional TCA cycle in *ATCS* line 6 and could be evidence of increased mitochondrial citrate export in this line. However, oxidative PPP fluxes in this line were also poorly determined. In the current model of *ATCS* line 6, a 33% increase in oxidative PPP would meet the demand for NADPH. This is within the bounds of the net oxidative PPP flux deviations.

The increase in TCA cycle flux in *ATCS* line 6 was predicted to cause excess CO₂ production. Redistribution of ¹⁴C from [U-¹⁴C]glucose into CO₂ by this line was not measured to be different to control lines. However, the model does not account for all sources of CO₂ (such as that created during lipid turnover, which could differ between lines). Nevertheless, increased CO₂ production by a heterotrophic organism would equate to lower CCE and, in the absence of increased respiration, slower growth. The growth rate of *ATCS* line 6 was reduced (Chapter 4.2.3), while O₂ uptake was no different to control lines. This seems to corroborate the increase in CO₂ output deduced by MFA. By extension, the normal growth uptake phenotype of *ATCS* line 16 would appear to substantiate the unchanged TCA cycle fluxes deduced by MFA for this line.

5.3.6. Phenotypic differences between *ATCS* transgenic lines may be due to mistargeting of the *ATCS* protein

Although the CS activity of *ATCS* line 16 was not as high as line 6, it was still double the control activity. This raises the question of why *ATCS* line 16 did not have altered TCA cycle fluxes. It is unlikely that the increased TCA cycle flux in *ATCS* line 6 is a pleiotropic effect unrelated to the increased extractable CS activity. While it is possible that an increase in TCA cycle flux in *ATCS* line 16 could be disguised by the flux deviations, any increase in this line would be small relative to the increase in *ATCS* line 6 (in the order of 1.1 times that of control line 6, which had the highest control CS flux, assuming that CS flux of *ATCS* line 16 is at the upper end of its deviation. This is compared to the 1.4 times increase in best-fit CS flux of *ATCS* line 6). Given that the degree of control exerted by an enzyme on pathway flux decreases as the enzyme becomes more abundant (other things being unchanged) (Fell, 1997), *ATCS* line 16 would have been expected to have flux through CS increased by a magnitude of at least 1.35, as calculated by dividing the relative increase in CS activity of *ATCS* line 16 (compared to controls = 1.1) by the relative increase in CS activity of *ATCS* line 6 (compared to controls = 1.4) and multiplying by the relative increase in flux through CS in *ATCS* line 6 (compared to control line 6 = 0.45).

The absence of the expected effect of increased CS activity on TCA cycle flux in *ATCS* line 16 implies that *ATCS* in *ATCS* line 16 was either not correctly targeted to the mitochondrion or was not forming the correct associations with other enzymes in the mitochondrion. The consistency with which increased CS activity was extractable from *ATCS* line 16 (Chapter 4.2.2) refutes alternative possibilities of an error in transgene integration or a regulatory mechanism affecting protein translation. It also disproves the possibility of *ATCS* being misfolded. Overexpression can lead to mislocalisation of the gene product (Sparkes et al., 2006; Geldner et al., 2007; Burén et al., 2012; Denecke et al., 2012). However, it would be

surprising for ATCS to be successfully targeted to the mitochondrion in one line and not in the other, and mistargeting would be expected to occur to a greater degree in the line with higher ATCS production. Also a doubling of extractable ATCS activity does not suggest that expression of the *ATCS* transgene was exceptionally high. Nevertheless, overexpression-induced mistargeting seems to be the most plausible explanation for the lack of effect on TCA cycle flux of increased extractable CS activity in *ATCS* line 16. Alternative possibilities are that a point mutation occurred in the mitochondrial targeting presequence of *ATCS* in *ATCS* line 16, which could prevent ATCS being correctly targeted to the mitochondrion (Ambard-Bretteville et al., 2003; Duby et al., 2003), or that chromosomal integration of *ATCS* disrupted the expression of one or more other genes such that the effect of increased CS activity on TCA cycle flux was negated. However, it would be highly unlikely for insertional mutagenesis to result in such a near-WT phenotype, making this possibility seem implausible.

A final possible explanation of the differences in TCA cycle flux between the two *ATCS* lines is that a threshold of heterologous enzyme activity could be required to be exceeded before a change in flux is realised. For instance, allosteric inhibition of the endogenous CS could oppose any increase in flux favoured by the increased total CS levels. If that were happening, a more than doubling of total CS protein may be required to achieve an altered flux. This would correlate with the lack of detectable flux change through CS associated with an approximate doubling of total extractable CS in *ATCS* line 16, whereas the 2.4 times the extractable control CS activity detected in *ATCS* line 6 was associated with a CS flux of 1.4 times the control flux. However, there is an 83% amino acid sequence identity between ATCS and the endogenous CIT1 (when aligned using BLASTP (Altschul et al., 1990)). Therefore, it seems unlikely that the two proteins would be subject to allosteric regulation that is sufficiently different for this explanation to be correct.

The hypothesis that ATCS was mistargeted in *ATCS* line 16 could have been tested had the genetic engineering approach been different. Inclusion of a fluorescent tag within the DNA sequence would have allowed localisation of the foreign protein to be determined. This was an approach that was considered during the early phase of the project but was deemed unwise due to the potential for the fluorescent tag itself to cause mistargeting (Lisenbee et al., 2003; Marinova et al., 2007) or misfolding (Moore and Murphy, 2009) of the protein.

Both *ATCS* lines studied in detail had greater levels of end-of-pathway sterols than controls, while levels of intermediate sterols were reduced (Chapter 4.2.6). The similarities between the two lines in this respect seem to contradict the argument for ATCS mislocalisation in *ATCS* line 16, as it indicates that some altering of sterol biosynthetic flux did occur in this line. However, it may have been the case that not all ATCS was mistargeted and that the TCA cycle flux did in fact alter in this line by an undetectable degree. (Best-fit CS flux would have had to be at least 1.3 times that of control line 6 for the difference to have been statistically significant.) Supporting this hypothesis is the observation of a slight increase in O₂ uptake by *ATCS* line 16 in Chapter 4.2.4. Furthermore, the absence of total sterol yield increases in *ATCS* line 16, when considered against the 18% increase in yield in *ATCS* line 6, suggests that the degree of any change in TCA cycle flux was small.

5.3.7. Changes in tricarboxylic acid cycle fluxes have been identified in other systems by steady-state metabolic flux analysis

An approximate doubling of TCA cycle flux was induced in *Arabidopsis* heterotrophic cell suspension cultures by culturing at increased O₂ concentrations (Williams et al., 2008), and *Arabidopsis wrinkled1* mutant embryos had approximately four times the TCA cycle flux of

WT embryos (Lonien and Schwender, 2009). In comparison to these studies, the 1.4 times increase in TCA cycle flux achieved in *ATCS* line 6 seems small. However, the increase in TCA cycle flux in Arabidopsis cell cultures grown at increased O₂ concentration was accompanied by comparable increases in glucose uptake and biosynthetic fluxes (Williams et al., 2008), whereas the BY-2 cell lines all had similar glucose uptake. Arabidopsis *wrinkled1* mutant embryos did not have large changes in uptake flux (Lonien and Schwender, 2009), but the relevance of this study to BY-2 metabolism is questionable given differences in uptake fluxes and effluxes between the two systems. For instance, CCE of WT Arabidopsis embryos was 80% compared to about 60% in BY-2 cell cultures. As already discussed here and elsewhere (Chen and Shachar-Hill, 2012), there is a strong link between CCE and TCA cycle flux, and the high CCE of WT Arabidopsis embryos is indicative of the low TCA cycle flux in this system. Therefore, it is not clear if it would be possible to increase TCA cycle flux further in BY-2 cells without substantially decreasing CCE, which would further impact on growth rate.

5.3.8. Conclusions

¹³C steady-state MFA of BY-2 cells provided well-determined values of TCA cycle flux and established BY-2 as an excellent system on which to perform MFA. Duplicated glycolytic and PPP fluxes were determined less well due to the choice of labelling experiments and analytical techniques. The MFA results do not consistently reveal altered CS flux in cell lines with increased extractable CS activity, but significantly increased CS flux was identified in the line with highest extractable CS activity. Problems with trafficking of the ATCS protein into mitochondria may explain the absence of increased CS flux in the other line. The increase in TCA cycle flux brought about by increased CS activity in *ATCS* line 6 did not cause

significant alterations in biomass composition but probably caused a slight reduction in CCE, which may explain the slower growth rate of this line identified in Chapter 4.

Chapter 6. Increasing the demand for acetyl-CoA by increasing activity of mevalonate pathway enzymes

6.1. Introduction

6.1.1. Objectives

The objectives of this chapter were:

1. to increase the yield of sterols in Bright Yellow 2 (BY-2) tobacco (*Nicotiana tabacum*) cells by manipulating the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) in the mevalonate pathway of isoprenoid biosynthesis and thereby to increase the demand for mitochondrial citrate export;
2. to couple this modification of sterol biosynthesis to transgenic *ATCS* lines with increased citrate synthase activity in order to further increase sterol yields.

6.1.2. Control of isoprenoid biosynthesis

Flux through primary metabolites such as acetyl-CoA (the precursor for sterol biosynthesis) occurs at a sufficiently high rate that under normal circumstances the supply of a primary metabolite to a native secondary biosynthetic pathway is unlikely to be a significantly limiting flux through that pathway. One way that the precursor supply is likely to become limiting is if the demand from the secondary pathway is significantly increased. This can be achieved by increasing the activity of secondary pathway enzymes that exert a large degree of control over the formation of the desired product. Coupling such an increase in the activity of a relevant

secondary pathway enzyme to the increases in citrate synthase (CS) activity achieved in Chapter 4 may allow for further product yield increases to be achieved by causing a redirection of some of the increased tricarboxylic acid (TCA) cycle flux identified in Chapter 5 into sterol biosynthesis.

Sterols are products of the mevalonate isoprenoid biosynthesis pathway, which converts acetyl-CoA molecules to isopentenyl diphosphate (IPP) (Figure 6.1). A further series of reductive reactions convert IPP and its isomer dimethylallyl diphosphate (DMAPP) to squalene, the first committed intermediate in sterol biosynthesis. A tobacco mutant (LAB 1-4) exhibiting a high sterol phenotype was found to have increased HMGR activity (Maillot-Vernier et al., 1991; Gondet et al., 1992; Gondet et al., 1994; Schaller et al., 1995), and over-expression of *HMGR* in tobacco (Chappell et al., 1995; Harker et al., 2003b; Holmberg et al., 2003) and other plant species (Enfissi et al., 2005; Muñoz-Bertomeu et al., 2007) caused increased accumulation of sterols. Furthermore, HMGR activity responds positively to depletion of sterols (Wentzinger et al., 2002). This led HMGR to be considered as the primary control point of IPP and sterol biosynthesis. It catalyses the following irreversible reaction (Brooker and Russell, 1975; Bach and Lichtenthaler, 1984):



Arabidopsis (*Arabidopsis thaliana*) has two HMGR genes (*HMG1* and *HMG2*) (Enjuto et al., 1994), whereas varying numbers exist in other plant species, including at least nine in potato (*Solanum tuberosum*) (Stermer et al., 1994). *Arabidopsis* HMGRs are both localised to the endoplasmic reticulum (Enjuto et al., 1994; Leivar et al., 2005). Tobacco also has two HMGR genes. *HMG1* is constitutively expressed (Learned, 1996), whereas *HMG2* has stress-induced expression (Merret et al., 2007).

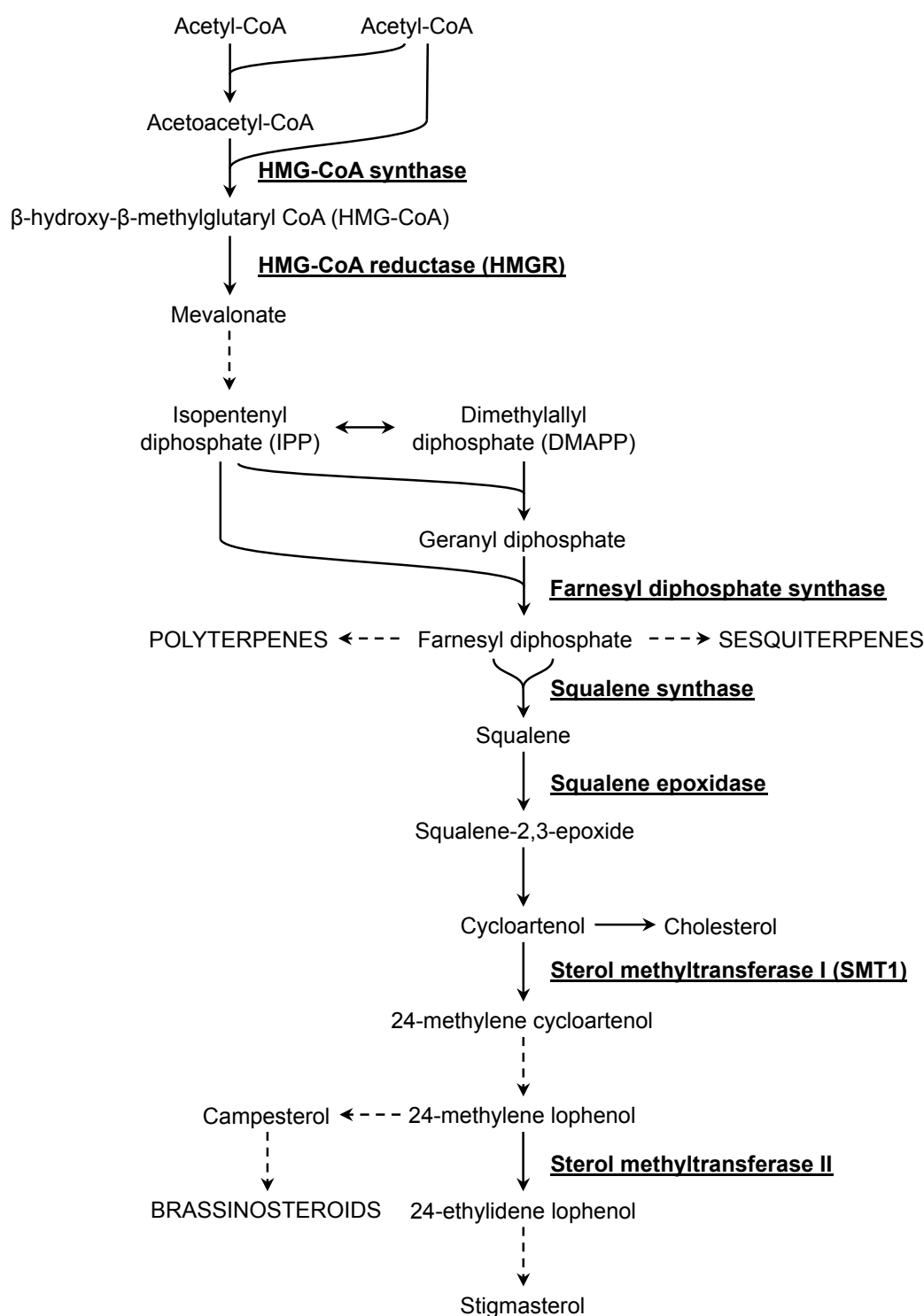


Figure 6.1. Overview of isopentenyl diphosphate (IPP) and sterol biosynthesis in plants.

IPP is synthesised from acetyl-CoA in the cytosol. This is then successively added to DMAPP to produce the 15C farnesyl diphosphate. Reductive head-to-head condensation of two molecules of farnesyl diphosphate creates squalene, the first committed step in sterol biosynthesis. This pathway also results in the biosynthesis of sesquiterpenes, polyterpenes and brassinosteroids. Enzymes exhibiting significant control over sterol biosynthesis are highlighted. Dashed arrows represent multiple steps.

Transformation of tobacco plants with rubber tree (*Hevea brasiliensis*) *HMG1* led to up to six-fold increases in sterol levels, with the largest increases being in sterols formed early in the sterol biosynthetic pathway, particularly cycloartenol (Schaller et al., 1995). Morphology was unchanged. Transformation of tobacco plants with a truncated hamster *HMGR* also resulted in increased total sterols (Chappell et al., 1995). Despite this research on tobacco plants, there are no published accounts of *HMGR* overexpression in BY-2 cells, although inhibition of squalene synthase and squalene epoxidase both triggered increased *HMGR* activity without obvious negative effects on cell viability (Wentzinger et al., 2002).

Sterol profiles arising from increased *HMGR* activity varied between studies, but intermediates, especially cycloartenol, tended to increase more than 4-desmethyl end-of-pathway sterols. This led to the identification of the 24-methylation of cycloartenol by sterol methyltransferase I (*SMT1*) (Figure 6.1) as a step exerting significant control over flux into downstream sterols (Holmberg et al., 2003). Transforming tobacco plants with both an N-terminal truncated *HMG1* fragment and *SMT1* resulted in higher sterol yields than in single transformants. Increasing *SMT1* activity on its own was only about 20% as effective at increasing total sterol yields as manipulating *HMGR*, and this may be partly due to the lifting of negative feedback imposed upon *HMGR* by cycloartenol (Holmberg et al., 2002). However, the yield increase provided by *SMT1* doubled when co-expressed with *HMG1*, and the proportion of end-of-pathway sterols was increased at the expense of cycloartenol (Harker et al., 2003b).

Other strategies investigated for direct effects on sterol yield include over-expression of HMG-CoA synthase (Wang et al., 2012). This increased sterol yields in *Arabidopsis*, but only marginally, and these increases may be more attributable to induced expression of downstream genes including those encoding *HMG1*, *HMG2* and sterol methyltransferase II. Over-expression of squalene synthase also led to a doubling of stigmasterol and sitosterol

content in *Eleutherococcus senticosus* (Seo et al., 2005). Farnesyl diphosphate (FPP) synthase overexpression resulted in up to three-fold increases in sterol yields in *Centella asiatica* hairy root cultures (Kim et al., 2010), but sterols were unaffected in Arabidopsis overexpressing FPP synthase (Masferrer et al., 2002). Furthermore, these plants grew poorly, probably due to mevalonate depletion leading to insufficient levels of the cytokinin 2-isopentenyladenine.

HMGR was chosen as the candidate for over-expression due to the large amount of knowledge about its regulation (Learned and Fink, 1989; Omkumar and Rodwell, 1994; Campos and Boronat, 1995; Dale et al., 1995; Harker et al., 2003a; Harker et al., 2003b; Merret et al., 2007; Leivar et al., 2011) in addition to the number of studies that demonstrate that it exerts significant control over the accumulation of sterols in plants.

6.2. Results

6.2.1. Generation of P_{35S}-*HMGI* and control expression vectors

The pBI121-*HMGI* vector containing the full length Arabidopsis *3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE 1* (*HMGI*; At1g76490) complementary DNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Enfissi et al., 2005) was kindly provided by Dr Eugenia Enfissi, Royal Holloway, University of London. This vector was amplified in *E. coli* DH5 α before the insert was verified by sequencing (Appendix 6.1).

pBI121 confers kanamycin resistance to transformed plants through expression of *nptII*, (*neomycin phosphotransferase II*) under the transcriptional control of a *nos* (*nopaline synthase*)

promoter and terminator. An appropriate control vector was created by recombining *mCherry* from pENTR201-*mCherry* (resulting from addition of *mCherry* to pDONR201 in Chapter 4.2.1) into pK7WG2 (Karimi et al., 2002) by Gateway recombination. This created pK7WG2-*mCherry*, which confers to transformed plants the expression of *mCherry* under the control of *P_{CaMV35S}* and *nptII* under the control of the *nos* promoter and terminator. The pK7WG2-*mCherry* insert was confirmed by sequencing (Appendix 6.2).

Agrobacterium tumefaciens was transformed with either the pK7WG2-*mCherry* (control) or pBI121-*HMG1* (*HMG1*) vector. Polymerase chain reaction (PCR) of resulting colonies confirmed successful transformation (Figure 6.2). *HMG1* and control agrobacteria were then used to transform wild-type (WT) BY-2 cultures. Transformed cells were plated on solid BY-2 medium containing 100 µg ml⁻¹ kanamycin as selective antibiotic. Overviews of the transfer DNA (T-DNA) segments are presented in Figure 6.3.

6.2.2. Screening transgenic lines for increased sterol yields

40 kanamycin-resistant calli resulting from transformation with the *HMG1* construct and three resulting from transformation with the control construct were introduced into suspension medium. 14 of the *HMG1* lines failed to grow in suspension. The remaining 26 *HMG1* lines and the three control lines were screened for sterol content after allowing cultures to acclimate to suspension culturing for a minimum of five subculture cycles. Seven *HMG1* lines (3, 10, 16, 21, 28, 34 and 38) had greater than 50% increases in total sterol dry weight (DW) content at seven days after subculture compared to the control average (Figure 6.4a). *HMG1* lines 3, 16 and 34 had stopped dividing in suspension by 21 weeks of suspension culture. *HMG1* lines 10,

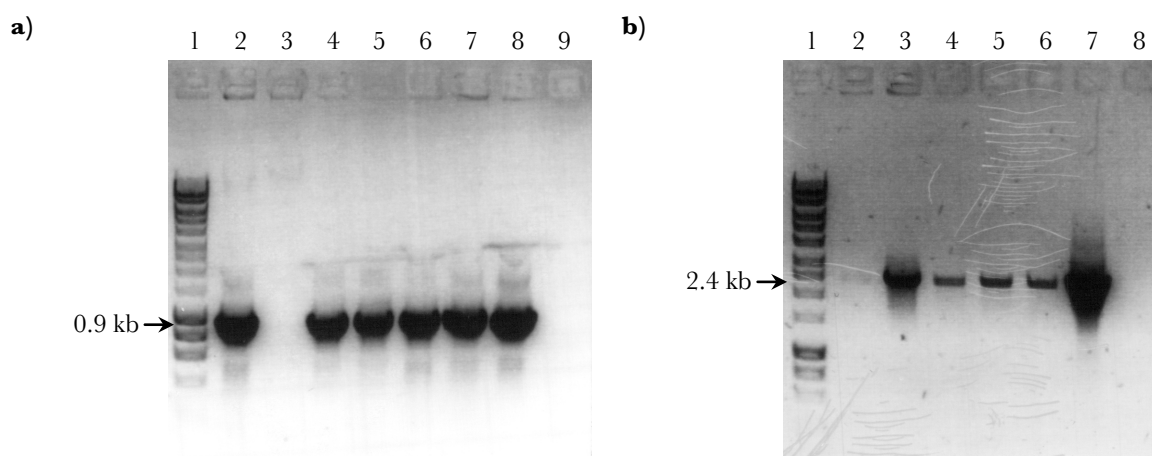
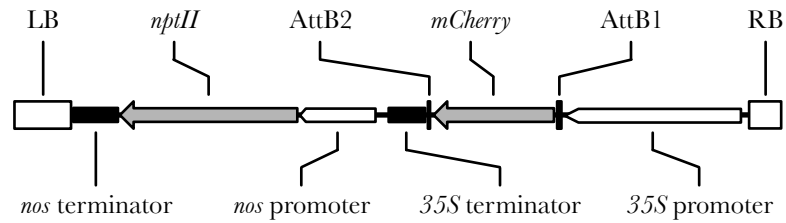


Figure 6.2. PCR analysis of pK7WG2-*mCherry* and pBI121-*HMG1* expression vectors.

a) PCR of pK7WG2-*mCherry* insert using p7WG2-ins-F and p7WG2-ins-R primers. Expected product size of 919 bp. Lanes 2-7 contain colony PCRs of transformed *A. tumefaciens* GV3101:pMP90; lane eight is a pK7WG2-*mCherry* purified plasmid positive control that had been previously verified by sequencing of the insert. Lane 9 was a negative control of the PCR reaction without template. **b)** PCR of pBI121-*HMG1* insert using pBI121-ins-F and pBI121-ins-R primers. Expected product size of 2,387 bp. Lanes 2-6 are colony PCRs of transformed *A. tumefaciens* GV3101:pMP90. Lane 7 is a purified pBI121-*HMG1* plasmid, the insert of which had been confirmed by sequencing. Lane 8 is a PCR negative control. Lane 1 of each gel contains HyperLadder I (Bioline Reagents Ltd).

a)



b)

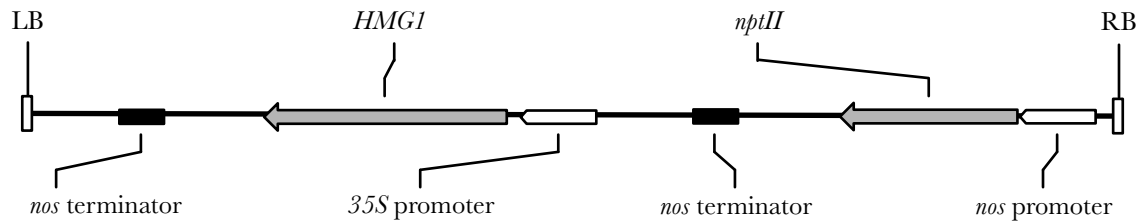


Figure 6.3. Overview of P_{35S} -*HMG1* and control transfer DNAs.

a) *mCherry* (control) T-DNA segment (4.1 kb) from pK7WG2-*mCherry*. **b)** *HMG1* T-DNA segment (6.6 kb) from pBI121-*HMG1*.

Abbreviations: **AttB1** and **AttB2**, Gateway recombination sites; ***HMG1***, Arabidopsis *HMG1* coding sequence from complementary DNA; ***nos***, nopaline synthase; ***nptII***, neomycin phosphotransferase; **LB** and **RB**, T-DNA borders.

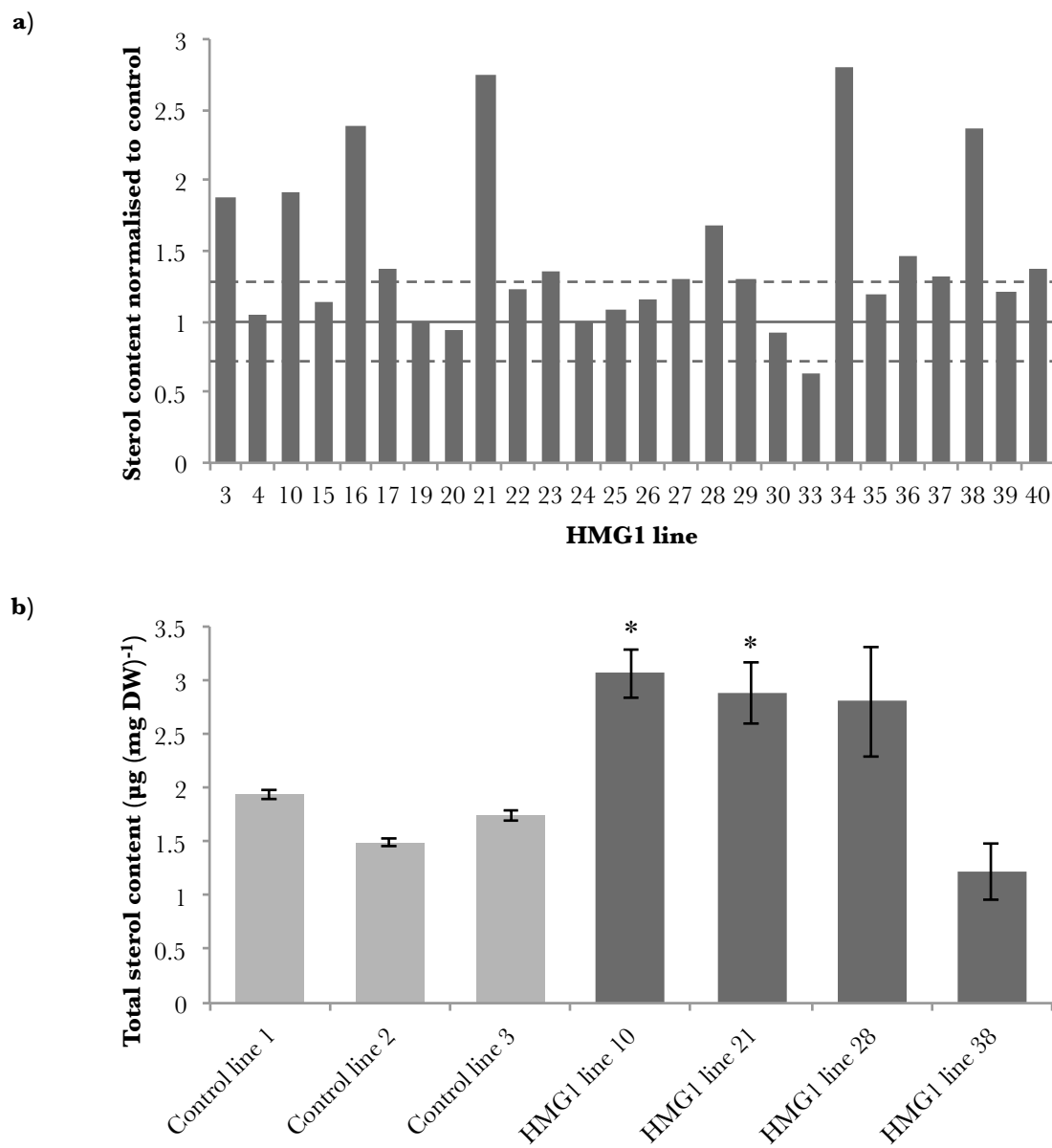


Figure 6.4. Total sterol content of transgenic *HMG1* BY-2 lines.

a) Total sterols were extracted in four batches from seven-day-old cell suspension cultures of 26 transgenic *HMG1* BY-2 lines. Sterol levels are normalised to the mean of three transgenic control lines. Each bar represents a single sample. Dashed lines indicate standard error (SEM) of the control lines. **b)** Further analysis of *HMG1* lines 10, 21, 28 and 38 revealed significant increases in total sterol content of lines 10 ($p < 0.01$) and 21 ($p < 0.05$) relative to each of the control lines (student's t tests). *HMG1* lines 10 and 21 had $76\% \pm 13\%$ and $67\% \pm 16\%$ higher sterol content than the control average, respectively (mean $\pm$ SEM). Error bars indicate SEM of three biological replicates.

21, 28 and 38 were further analysed for sterol content (Figure 6.4b). The results indicated that *HMG1* lines 10 and 21 had significantly greater total sterol content at seven days than each of the three control lines (student's *t* test, $p < 0.05$), with yields increased to 1.8 ± 0.1 and 1.7 ± 0.2 times the control average, respectively.

The sterol profiles of *HMG1* lines 10 and 21 were investigated further (Figure 6.5). Minor sterols (cycloartenol, cholesterol, 24-methylene-cholesterol, Δ^5 -avenasterol and fucosterol) were unchanged in either line. Sitosterol yields were 2.0 ± 0.2 and 2.3 ± 0.2 times those of the control average in *HMG1* line 10 and 21, respectively ($p < 0.01$ relative to each control line, student's *t* tests). Campesterol levels in *HMG1* line 10 were 2.2 ± 0.2 times those of the control average ($p < 0.05$ relative to each control line). *HMG1* line 21 campesterol levels also tended towards being increased, at 1.7 ± 0.2 times the control average levels, but were not significantly different to control line 1. Stigmasterol levels were unchanged in both lines.

6.2.3. Growth analysis of transgenic *HMG1* and control BY-2 cultures

Overproduction of sterols in *HMG1* lines 10 and 21 had no discernable effect on suspension culture growth rate. These lines and the control lines each had a lag phase of approximately two days followed by rapid growth until five days after subcultures and reached a fresh weight (FW) of 270-300 mg ml⁻¹ by 10 days after subculture (Figure 6.6). However, *HMG1* cultures appeared greyer than controls by seven days after subculture, by which time stringy amalgamations of cells had also formed in these cultures (Appendix 6.3).

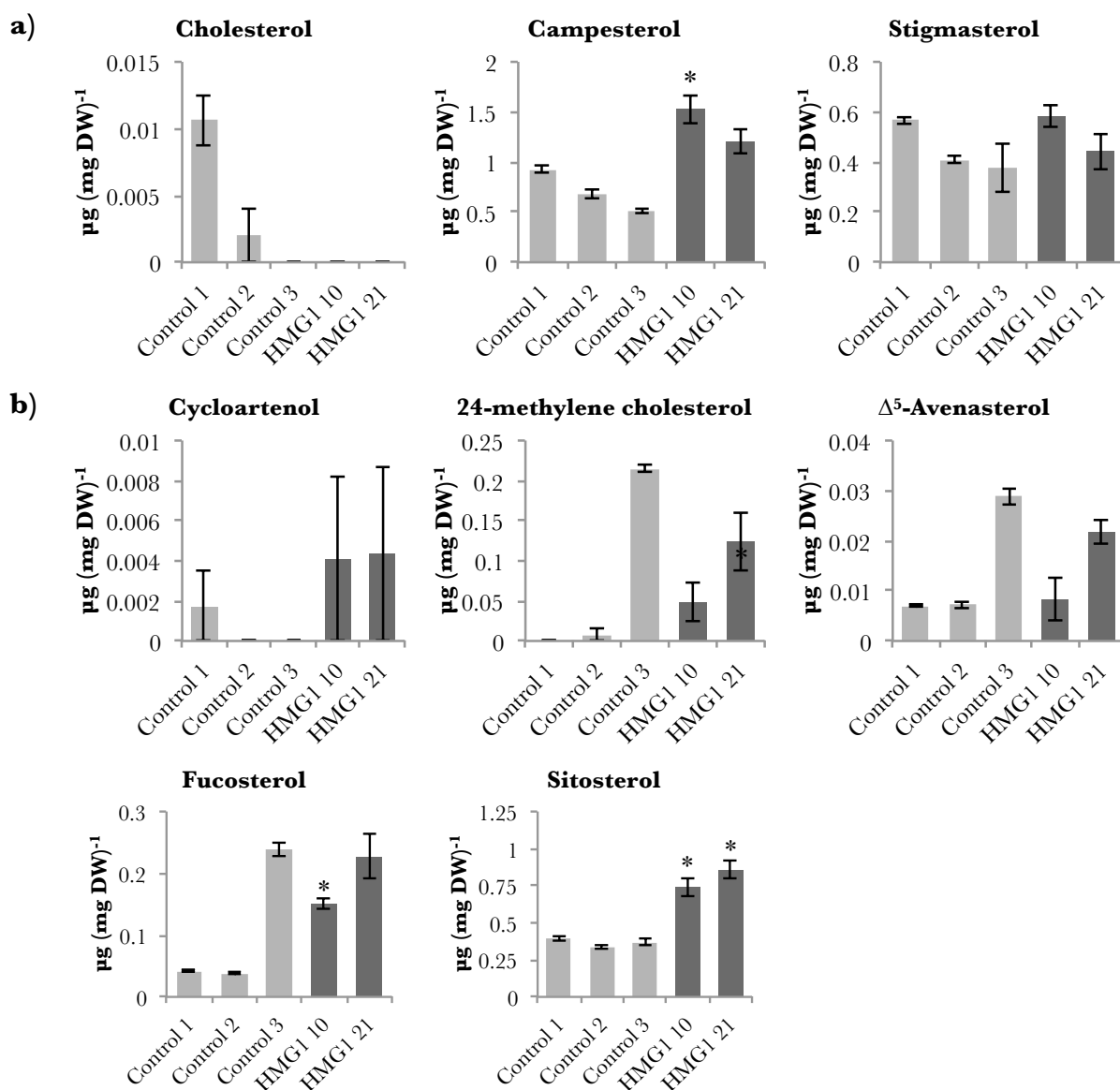


Figure 6.5. Quantification of specific sterols in transgenic *HMG1* and control BY-2 cell lines.

DW content of **a)** end-product sterols, and **b)** pathway intermediate sterols. The order in which they are presented is based on the proximity to cycloartenol in the sterol biosynthesis pathway. Error bars represent SEM, $n = 3$. *HMG1* measurements that are significantly different to each of the control lines (significant after Bonferroni correction with initial critical p-value of 0.05, student's t test) are denoted by "*". There were no significant differences between the two *HMG1* lines following Bonferroni correction for multiple comparisons.

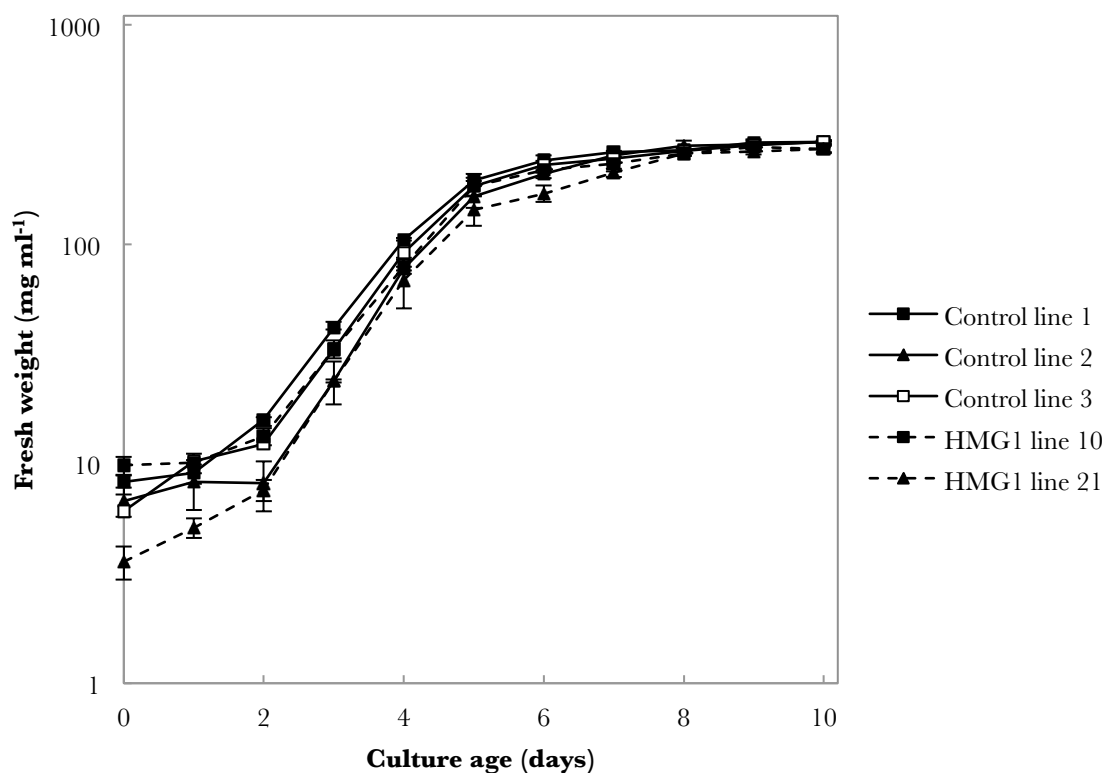


Figure 6.6. Growth of transgenic *HMG1* and control BY-2 cell lines in suspension medium.

FW was quantified in aliquots taken from cell suspension cultures at 24 h intervals. There were no consistent differences between *HMG1* lines and control lines. Error bars indicate SEM of a minimum of three biological replicates.

6.2.4. Assay of HMGR activity in microsomal extracts from transgenic *HMGI* and control BY-2 cultures

HMGR activity was measured in microsomal extracts from the control lines and *HMGI* lines 10 and 21 20 sub-culture cycles after detailed assays of sterol levels. A maximum of six microsomal extractions could be performed at any time due to limited equipment capacity. This meant that extractions had to be performed in batches of single replicates per transgenic line. Microsomal extraction was performed on freshly harvested BY-2 cultures, which, together with restricted equipment capacity and availability, meant that HMGR activity was assayed at two-week intervals. Despite these limitations and the inherent variability of the assay, discussed in Chapter 3.3.6, HMGR assays were considered to be a more appropriate method for assessing transformants than assaying *HMGI* transcript abundance, as transcript abundance is a poor indicator of enzyme activity (Gibon et al., 2004).

HMGR activity of each batch was expressed relative to the control average of that batch to eliminate batch effects (such as differences in microsomal extraction efficiency) and to allow comparisons between batches. HMGR activity in *HMGI* lines initially appeared to be greater than in control lines, but measured activity in *HMGI* line extracts dropped over the course of the experiment relative to the controls (Figure 6.7a). This led to there being no detectable differences in HMGR activity between *HMGI* and control lines when normalised by batch (Figure 6.7b) or when absolute HMGR activity was considered (Appendix 6.4, student's *t* tests).

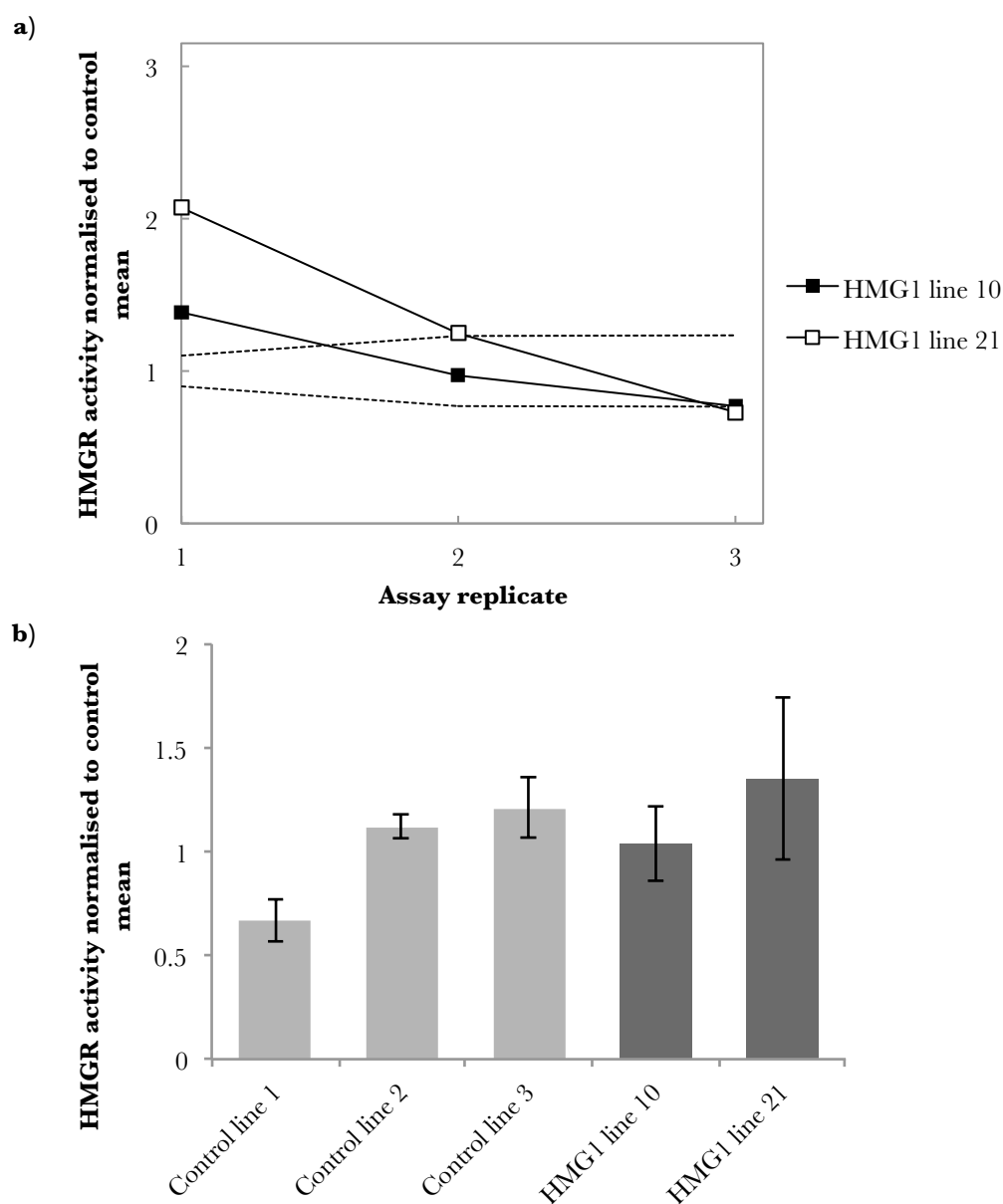


Figure 6.7. HMGR activity of transgenic *HMG1* and control BY-2 lines.

a) Time-course of HMGR activity in microsomal extracts. Assays were repeated on freshly harvested cultures at two-week intervals. HMGR activity was normalised within each batch to the average of the control values in order to control for variation in extraction efficiency between batches. Dashed lines indicate SEM of control values. **b)** Average HMGR activity in microsomal extracts in (a) relative to the control average. Error bars indicate SEM of the three biological replicates. Neither *HMG1* line had significantly different HMGR activity to any of the control lines (student's *t* tests). Absolute values from this experiment are plotted in Appendix 6.4.

6.2.5. Re-evaluation of sterol levels in transgenic *HMG1* and control BY-2 lines

The absence of detectable differences in extractable HMGR activity between *HMG1* and control lines led to the suspicion that transgene silencing was occurring in the *HMG1* lines. Transgene silencing was expected to result in *HMG1* line sterol levels returning to control values. Indeed, total sterol levels in cultures harvested after performing HMGR assays were no different between *HMG1* lines and controls (Figure 6.8a, compare to Figure 6.4). There were also no differences in the content of individual sterols except in the case of *HMG1* line 21 sitosterol content, which was at $80\% \pm 5\%$ of the control average ($p < 0.001$, significant after Bonferroni correction for multiple comparisons, student's *t* test; Figure 6.8b).

It was considered that the high sterol phenotype might be rescued by introducing transgenic calli, which had been maintained on kanamycin-containing BY-2 agar, into suspension culture. Calli were supplanted to suspension culture and grown until a cell density similar to seven-day-old WT cultures was achieved (approximately 10 days) before subculturing. New cultures were then harvested seven days after subculture for sterol extraction. There were no differences in total sterols between *HMG1* lines and controls (Figure 6.9a). There were significant differences between *HMG1* line 21 and the controls in campesterol and fucosterol content (a $0.2 \pm 0.0 \mu\text{g (mg DW)}^{-1}$ ($28\% \pm 5\%$) decrease and a $0.3 \pm 0.0 \mu\text{g (mg DW)}^{-1}$ (3.7 ± 0.2 -fold) increase, respectively; Figure 6.9b). However, these differences did not significantly affect total sterol yields. There were no differences in any measurements between *HMG1* line 10 and the controls.

To verify the loss of the high-sterol phenotype in calli, kanamycin-resistant calli were harvested for sterol extraction after four weeks growth on solid BY-2 medium containing

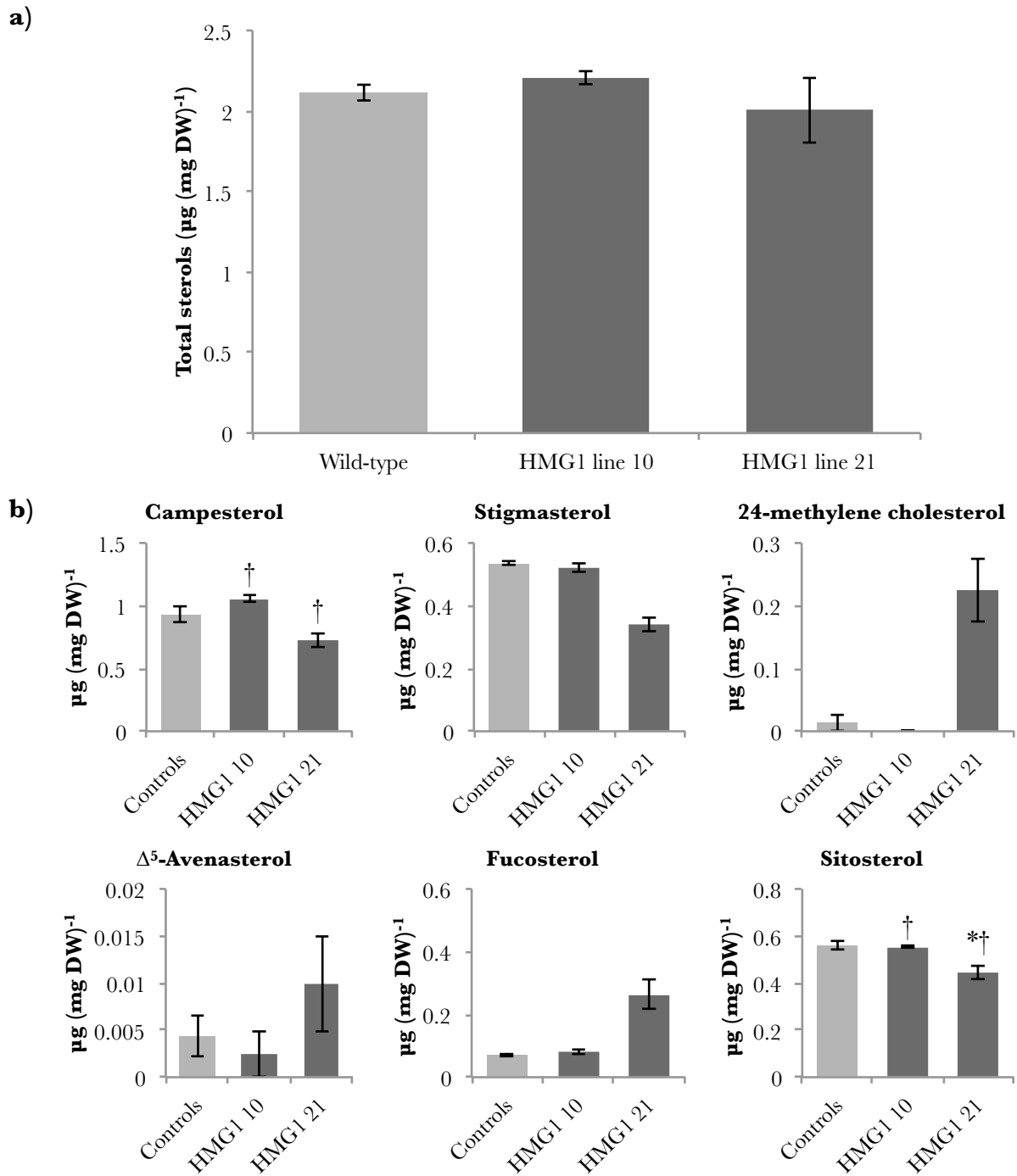


Figure 6.8. Sterol levels in transgenic *HMG1* and control BY-2 suspension cultures after suspected transgene silencing.

a) Total sterols. **b)** Individual sterols. Control measurements are the average of control lines 1, 2 and 3. Error bars represent SEM, $n = 3$. *HMG1* measurements that are significantly different to the control lines ($p < 0.0071$, critical p -value after Bonferroni correction for multiple comparisons, student's t test) are denoted by “*”. Measurements that are significantly different between the two *HMG1* lines are denoted by “†”.

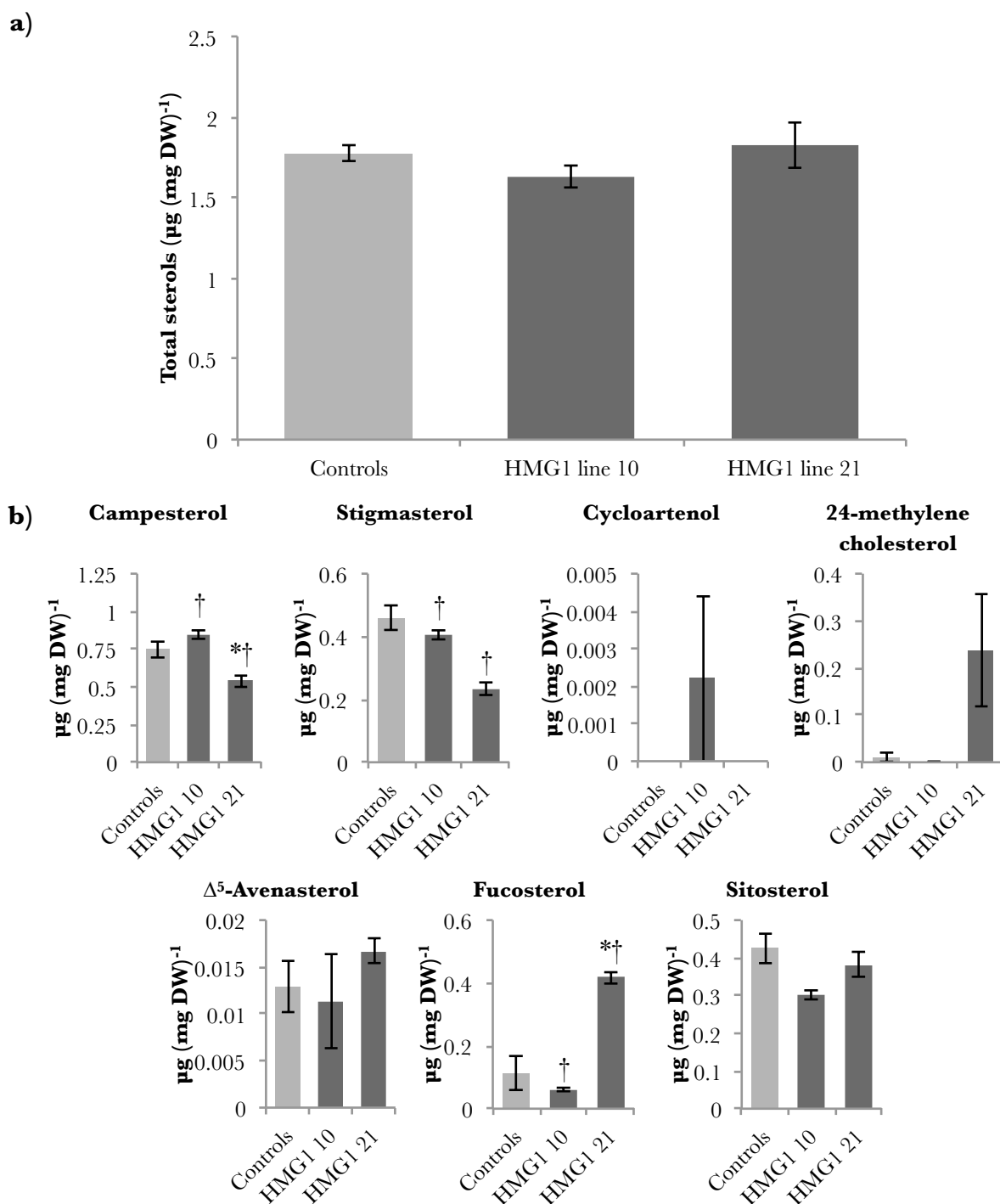


Figure 6.9. Sterol levels in transgenic *HMG1* and control BY-2 suspension cultures after resuspension of antibiotic-resistant calli.

a) Total sterols. **b)** Individual sterols. Control measurements are the average of control lines 1, 2 and 3. Error bars represent SEM, $n = 3$. *HMG1* measurements that are significantly different to the control lines ($p < 0.0071$, critical p-value after Bonferroni correction for multiple comparisons in b), student's t test) are denoted by “*”. Measurements that are significantly different between the two *HMG1* lines are denoted by “†”.

100 $\mu\text{g ml}^{-1}$ kanamycin. Sterols were then assayed from these calli. There were no differences in total sterols (Figure 6.10a) or in the levels of individual sterols (Figure 6.10b) between *HMG1* or control lines.

6.2.6. Transient transformation of tobacco leaves with *HMG1* and control constructs

Having established that sterol levels were no longer increased in *HMG1* BY-2 cell lines, transient transformation of tobacco leaves was attempted. This was done because transient transformation of tobacco leaves can be achieved over the course of days (unlike achieving transgenic BY-2 calli, which can take many weeks) and could allow the affects of several genetic manipulations to be easily studied simultaneously in the same cells. Transient expression in leaves also avoids the sometimes-lengthy procedure of introducing callus into suspension, during which transgene silencing can arise.

Tobacco leaf segments were infiltrated with agrobacteria containing either the *HMG1* construct or the control construct. After three days incubation leaf segments were freeze-dried and sterols were extracted. There were no differences in total or individual sterol contents of leaf areas between transformations with the control construct and those with the *HMG1* construct (Table 6.1).

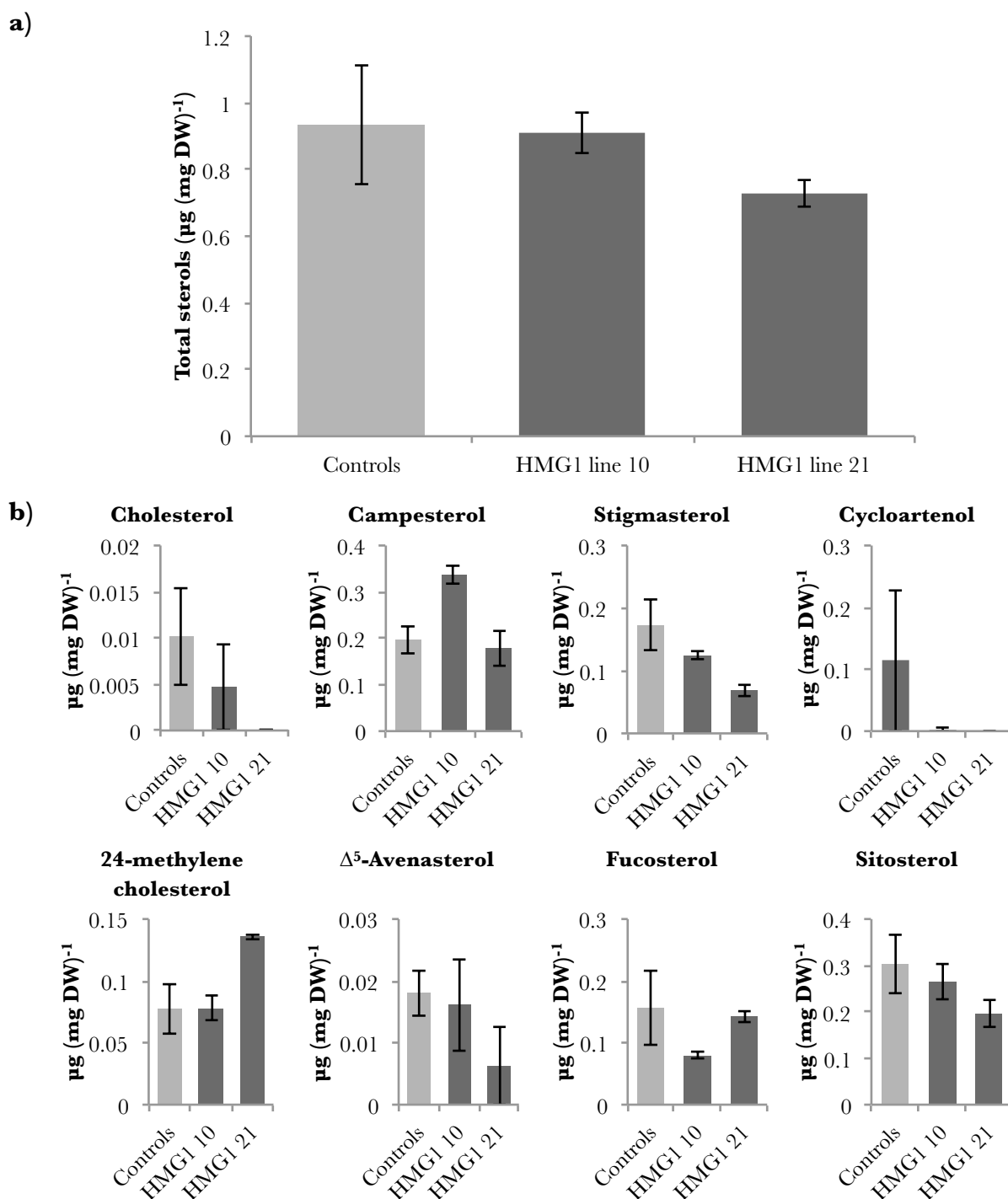


Figure 6.10. Sterols in transgenic *HMG1* and control BY-2 calli after suspected transgene silencing.

a) Total sterols, and **b)** individual sterols. Control measurements are the average of control lines 1, 2 and 3. Error bars represent SEM. Sterols were extracted from three individual calli for each transgenic line. There were no significant differences between *HMG1* lines and controls in either total or individual sterol levels (student's *t* tests).

Table 6.1. Sterol levels of tobacco leaf material after transient transformation with *HMG1* or the control construct.

Tobacco leaves were infiltrated with agrobacteria containing either pBI121-*HMG1* or the control vector (pK7WG2-*mCherry*). After three days leaf segments were excised, frozen in liquid nitrogen, lyophilised and sterols were extracted. The results indicate average sterol content of infiltrated segments from a single tobacco plant $\pm$ SEM. Three leaves were used for the infiltration and were divided in half down the midrib into control and *HMG1* zones. There were no statistically significant differences in either individual or total sterol content of the two conditions (student's *t* test).

	Sterol content (ng (mg DW) ⁻¹)	
	Control	<i>HMG1</i>
Cholesterol	122.0 $\pm$ 19.8	113.5 $\pm$ 11.0
24-methylene cholesterol	7.2 $\pm$ 3.7	19.0 $\pm$ 2.7
Campesterol	257.1 $\pm$ 38.5	249.4 $\pm$ 27.3
Stigmasterol	513.7 $\pm$ 71.2	487.4 $\pm$ 52.0
Sitosterol	251.6 $\pm$ 37.1	224.9 $\pm$ 24.0
Fucosterol	49.1 $\pm$ 6.8	65.6 $\pm$ 8.6
Cycloartenol	93.0 $\pm$ 13.3	78.2 $\pm$ 9.5
Total	1293.6 $\pm$ 190.3	1238.1 $\pm$ 133.3

6.2.7. Repeat transformation of BY-2 cells with pBI121-*HMG1* and pK7WG2-*mCherry* vectors

Transformation of WT BY-2 cells with the *HMG1* and control constructs was repeated. Transformations of *ATCS* line 6, which had the highest extractable CS activity in Chapter 4 and highest TCA cycle flux in Chapter 5, was also transformed with either of these constructs to enable any synergistic effects of the two manipulations to be determined. In each genetic background, kanamycin-resistant calli only arose after transformation with the control construct, indicating that transformation with the *HMG1* had failed to yield viable transformed cells.

It was considered that the lack of *HMG1* transformants could have been due to the loss of all or part of the pBI121-*HMG1* vector during agrobacterial culturing. To test this hypothesis plasmid DNA was isolated from the agrobacteria stock used to transform BY-2 cells. The plasmid isolate was used to transform *E. coli* DH5 α . Plasmids were then re-isolated from kanamycin-resistant *E. coli*. Agarose gel results following digestion of the isolated plasmids with PstI were consistent with the plasmid being pBI121-*HMG1* (Figure 6.11).

6.2.8. Further attempts at increasing HMGR activity/mevalonate pathway flux by genetic engineering.

Eukaryotic HMGR comprises a highly conserved C-terminal catalytic domain and a more variable N-terminal transmembrane domain. In hamster cells, degradation of HMGR is mediated by sterols and this sterol-mediated degradation is abolished in a truncated HMGR

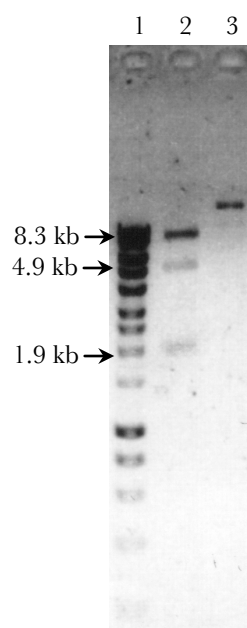


Figure 6.11. Confirmation of pBI121-*HMG1* by PstI restriction digestion.

Plasmids were purified from *A. tumefaciens* GV3101:pMP90 and transformed into *E. coli* DH5 α . Kanamycin-resistant *E. coli* colonies were cultured in suspension. Plasmids were purified from *E. coli* and analysed by PstI restriction digestion. Lane 1 contains HyperLadder I (Bioline Reagents Ltd), lane 2 contains the PstI-digested plasmid isolation (expected bands at 8.3 kb, 4.9 kb and 1.9 kb), and lane 3 contains the undigested plasmid.

that has the N-terminal domain deleted (Jingami et al., 1987). In mammals it seems that sterols recruit ubiquitination of HMGR (Song et al., 2005 and references therein). Although the structure of mammalian HMGR is different to plants (e.g. in number of membrane-spanning domains), there is some evidence suggesting that HMGR activity in plants (specifically potato) is mediated via proteolysis (Korth et al., 2000).

The effect of over-expression of an N-terminal truncated rubber tree HMGR (*tHMGR*) on HMGR activity and sterol yield has been compared against over-expression of a full-length HMGR in tobacco plants (Harker et al., 2003b). The full-length HMGR gave increases in seed sterols of up to 20% resulting from a 1.8-fold increase in HMGR activity, while the N-terminal truncated *H. brasiliensis* gave sterol increases of up to 2.7-fold resulting from increases in HMGR activity of up to 11-fold. This suggests that plant HMGR is regulated by the N-terminal in a similar manner to mammalian HMGR.

Based on this work, it was thought that transformation of BY-2s with an N-terminal truncated version of HMGR would result in larger increases in total sterol yield and HMGR activity than overexpression of the full-length gene, and would therefore cause greater demand on flux through primary metabolism. It was also thought that deregulation of HMGR activity would make any further manipulations to metabolism more likely to alter flux into sterols.

Generation of expression vectors with *HMGI*, an N-terminal truncated *HMGI* (*tHMGI*) and *mCherry* under the transcriptional control of the *UBQ10* promoter

Transformation vectors were created with the truncated *HMGI* gene (*tHMGI*), the full-length *HMGI*, or *mCherry* (control) under the control of a ubiquitin promoter (P_{UBQ10}) from *Arabidopsis* (Grefen et al., 2010). P_{UBQ10} was chosen because it is more similar to a native BY-2 promoter than P_{35S} and is weaker than P_{35S} (Grefen et al., 2010). Both of these features make

P_{UBQ10} less susceptible to silencing than the cauliflower mosaic virus 35S promoter (Grefen et al., 2010).

The pUB-*mCherry* vector was synthesised by Gateway recombination of *mCherry* from pENTR201-*mCherry* into pUB-DEST (Grefen et al., 2010). pUB-*HMG1* and pUB-*tHMG1* vectors were created by amplifying either the entire *HMG1* coding sequence or the final 1,289 bases of *HMG1* from pBI121-*HMG1*. ATG was included at the 5' end of the forward primer used when amplifying the 1,289 3' bases of *HMG1* in order to add a start codon to generate *tHMG1*. *HMG1* and *tHMG1* were T/A cloned into pCR8/GW/TOPO and were transferred to pUB-DEST via pDEST42 and pDONR201, as described in Chapter 4.2.1. Insert sequences for pUB-*mCherry*, pUB-*HMG1* and pUB-*tHMG1* were sequenced. pUB-*mCherry* and pUB-*HMG1* sequences were as expected. There was a synonymous substitution in the *tHMG1* coding sequence at 81 bp (T→C) in pUB-*tHMG1*. Vector sequences are presented in Appendices 6.5-6.7. Overviews of the T-DNA regions are shown in Figure 6.12.

Transformation of WT and a transgenic *ATCS* BY-2 line with pUB-*mCherry*, pUB-*HMG1* and pUB-*tHMG1* vectors

Prior to transformation with the pUB vectors, plasmids isolated from agrobacteria were transformed into *E. coli*, re-isolated, and subjected to analytical EcoRI digestion. The resulting digestion patterns were consistent with expectations for pUB-*mCherry*, pUB-*HMG1* and pUB-*tHMG1* vectors (Figure 6.13). These vectors were then transformed into the WT and *ATCS* line 6 backgrounds.

Concentrations of phosphinothricin ranging between 2 and 25 µg ml⁻¹ have been reported to select for phosphinothricin-resistant transgenic BY-2 cells (Ali et al., 2003; Du and Poovaiah, 2004; Kadota et al., 2004; van der Honing et al., 2010; Nakamura et al., 2010). 2, 4, 10, 25

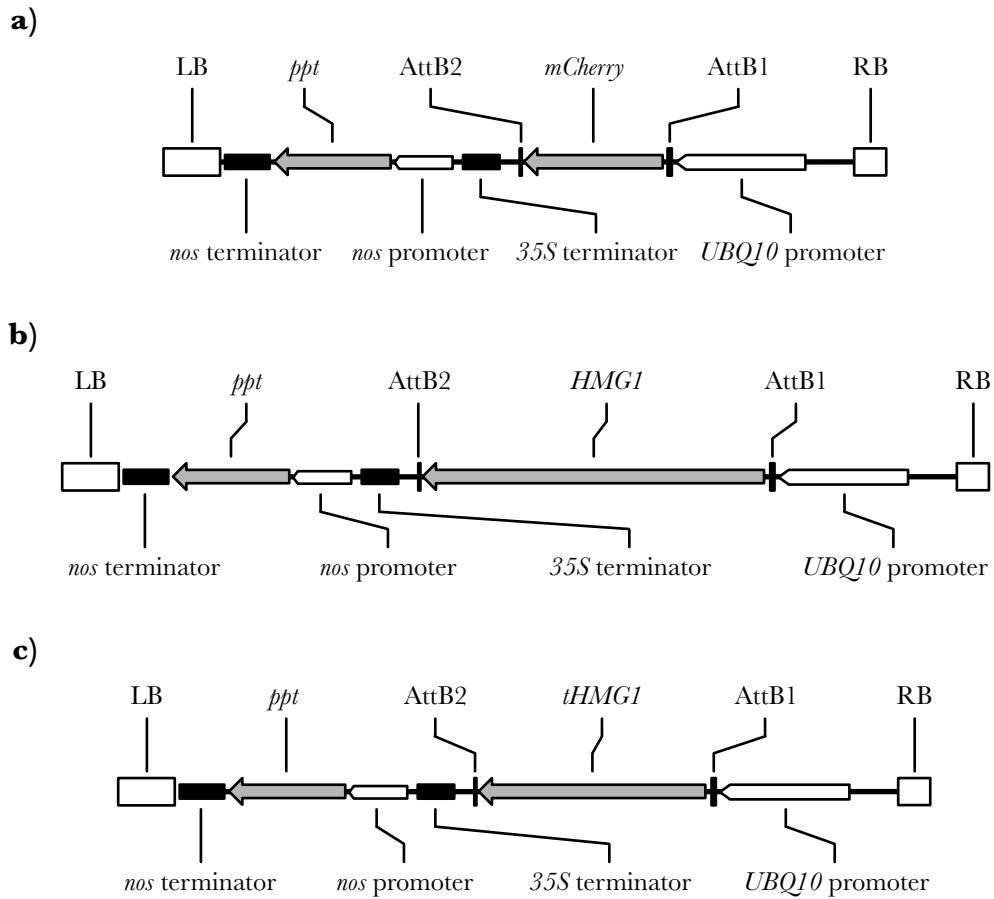


Figure 6.12. Schematic representations of P_{UBQ10} -*HMG1*, *tHMG1* and control transfer DNA segments.

a) *mCherry* (control) construct (3.7 kb). **b)** *HMG1* construct (4.8 kb). **c)** *tHMG1* construct (4.3 kb).

Abbreviations: **35S**, cauliflower mosaic virus 35S; **AttB1** and **AttB2**, Gateway recombination sites; **HMG1**, Arabidopsis *HMG1* coding sequence from complementary DNA; **LB**, T-DNA left border, **nos**, nopaline synthase; **ppt**, phosphinothricin N-acetyltransferase; **RB**, T-DNA right border; **tHMG1**, N-terminal truncated *HMG1*; **UBQ10**, Arabidopsis ubiquitin-10.

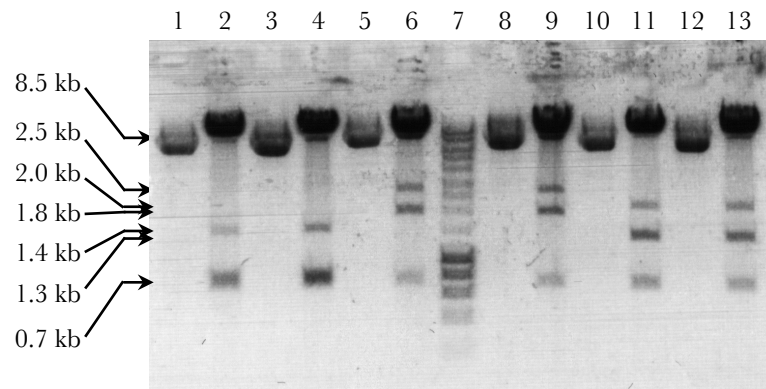


Figure 6.13. EcoRI restriction digestion of pUB-*mCherry*, pUB-*HMG1* and pUB-*tHMG1* vectors.

Plasmid DNA isolated from *A. tumefaciens* was transformed into *E. coli* DH5 α . Spectinomycin-resistant colonies were cultured overnight in suspension. Plasmid DNA from these cultures was digested by FastDigest EcoRI (Thermo Fisher Scientific Inc.) and analysed by agarose gel electrophoresis. Lane 7 contains HyperLadder I (Bioline Reagents Ltd). Undigested plasmid DNA is in lanes 1 and 3 (pUB-*mCherry*), 5 and 8 (pUB-*HMG1*), and 10 and 12 (pUB-*tHMG1*). These are each from different cultures. Adjacent lanes are EcoRI digests of that plasmid DNA. Expected digested fragment sizes were 0.7 kb (doublet) and 8.5 kb (pUB-*mCherry*), 0.7 kb, 1.8 kb and 8.5 kb (pUB-*HMG1*), and 0.7 kb, 1.3 kb and 8.5 kb (pUB-*tHMG1*). Each digest lane contained an additional band the size of the sum of the two smaller bands. This was the result of incomplete digestion, indicating that the plasmid had been cut twice rather than three times.

and 50 $\mu\text{g ml}^{-1}$ phosphinothricin was used as a selection. Transformation negative controls were performed whereby the cultures went through the transformation procedure but agrobacteria were not added. These negative controls, whether the WT or *ATCS* background, grew on solid BY-2 medium containing 2, 4 or 10 $\mu\text{g ml}^{-1}$ phosphinothricin. No antibiotic-resistant calli grew at the higher concentrations of 25 or 50 $\mu\text{g ml}^{-1}$ phosphinothricin. This meant that no transgenic lines were recovered from these transformations.

6.2.9. Attempts at increasing HMGR activity/mevalonate pathway flux by chemical elicitation.

Following the lack of success in attempts to genetically alter sterol biosynthetic flux, means of chemically eliciting a response were investigated. When choosing a compound to elicit an alteration in mevalonate/sterol biosynthetic flux, it was desirable to find one that would act somewhat specifically within that pathway and would be effective in BY-2 cells. For instance, *Sterol Methyltransferase 2* expression is regulated by various hormones including auxin, cytokinin and ethylene (Carland et al., 2002), but these hormones effect many other aspects of cell growth and division (Muday et al., 2012; Niklas and Kutschera, 2012). Similarly, gibberellic acid has been demonstrated to increase HMGR activity and sterol yield in cannabis (*Cannabis sativa*) leaves (Mansouri et al., 2009) but affects many cell processes.

Clomazone, a bleaching herbicide, can increase HMGR activity in maize, but only in light-grown maize (Ji and Hatzios, 1992), making it undesirable for use with BY-2 cultures grown in the dark. Farnesol also increases HMGR activity (Hemmerlin and Bach, 2000), but as it is a downstream intermediate of sterol biosynthesis it was also unfavourable. HgCl_2 increased

HMGR activity in potato tubers (Oba et al., 1985) and sweet potato (*Ipomoea batatas*) tubers (Ito et al., 1979), although the effects on sterol levels were not studied.

There is some evidence of stimulation of HMGR transcription by ethylene. For instance, *HMGR1* transcription increased in rubber tree after treatment with 2-chloroethane phosphonic acid (which generates ethylene *in vivo*), but the degree of increase was not measured (Chye et al., 1992) and there is no guarantee that BY-2 cells would respond in a similar manner. β -1,3-, 1,6-oligoglucan from the fungus *Alternaria alternata* elicited an increase in *HMGR* expression in BY-2 cells (Shinya et al., 2006), but this was part of a generalised defensive response, and the affect on HMGR activity was not measured.

The most promising candidate was considered to be cellulase from the fungus *Trichoderma viride*. Addition of this to tobacco KY-14 cell suspension cultures has been reported to elicit up to 29-fold increases in HMGR activity (Chappell et al., 1991). This treatment led to a redirection of mevalonate pathway flux from sterol biosynthesis into the biosynthesis of the isoprenoid phytoalexin capsidiol. This was possibly mediated by the inhibition of squalene synthase, which is known to lead to increased HMGR activity (Wentzinger et al., 2002). It was hypothesised that such a treatment of WT BY-2 cell cultures would have a similar effect, and that preliminary studies would lead to treatment of *ATCS* lines in the same manner, allowing comparison of the effect on mevalonate pathway flux between *ATCS* and control lines.

Elicitation with 0.4 mU ml⁻¹ *T. viride* cellulase

Total sterols were extracted from WT cultures subjected to 0.4 mU *T. viride* cellulase per ml cell culture for 10 h at four days after subculture. This corresponds to 0.1 μ g ml⁻¹, which caused a 90% reduction in the incorporation of [³H]mevalonate into sterols by tobacco KY-14 cells (Chappell et al., 1991). There were no differences in sterols between control BY-2 cultures or cultures subjected to 0.4 mU ml⁻¹ cellulase when comparing total or individual

sterol concentrations (Table 6.2). Capsidiol was not detected in CHCl_3 extracts from cell culture filtrate (CCF) or harvested cells from control or elicited cultures. There were no visual differences or differences in FW between the conditions, unlike tobacco KY-14 cultures, in which browning was noted in response to *T. viride* cellulase (Chappell et al., 1991).

***T. viride* cellulase concentration series**

Following the lack of changes in capsidiol and sterol levels after addition of 0.4 mU ml^{-1} cellulase, BY-2 cultures were subjected to a series of different concentrations of cellulase ranging from 0.04 to $4,000 \text{ mU ml}^{-1}$. $4,000 \text{ mU ml}^{-1}$ was lethal to the cells, causing cell lysis. Sterols and capsidiol were extracted from the remaining cultures. No capsidiol was detected in cell or CCF extracts. There were no differences in FW or appearance between the conditions. Sterol concentrations were greatest for the control culture without cellulase ($1.6 \mu\text{g (mg DW)}^{-1}$) and were lowest for 40 mU ml^{-1} cellulase ($1.0 \mu\text{g (mg DW)}^{-1}$; Figure 6.14).

Elicitation with 40 mU ml^{-1} *T. viride* cellulase

Following the initial results that suggested that a reduction in sterol biosynthesis might have been a response to 40 mU ml^{-1} *T. viride* cellulase, further experiments were conducted comparing the effects of 40 mU ml^{-1} cellulase against unelicited culture samples. Again, no capsidiol was detected in CCF or cell extracts. There were also no differences in total sterol levels or the levels of individual sterols between treatments (Table 6.3). Additional replicates were analysed for HMGR activity. These were harvested 5 h after addition of cellulase, at which point HMGR activity was found to be highest in KY-14 cultures (Chappell et al., 1991). There were no differences in HMGR activity between treated and untreated BY-2 cultures (Table 6.4).

Table 6.2. Effect of 0.4 mU ml⁻¹ *T. viride* cellulase on BY-2 cell culture sterol content.

Either 0.4 mU ml⁻¹ *T. viride* cellulase or no cellulase was added to four-day-old cell suspension cultures. Cultures were harvested after 10 h under standard culturing conditions and total sterols were extracted. Values refer mean $\pm$ SEM measurements of three biological replicates. There were no statistically significant differences in the levels of individual (following Bonferroni correction for multiple comparisons) or total sterols between the two conditions (student's *t* tests). No capsidiol was measured in chloroform extracts from these cultures. There were also no visual differences between cultures treated with cellulase and controls, and there were no differences in FW between the conditions.

	Sterol content (ng (mg DW) ⁻¹)	
	0 mU ml ⁻¹ cellulase	0.4 mU ml ⁻¹ cellulase
Cholesterol	7.6 $\pm$ 0.8	11.6 $\pm$ 0.4
24-methylene cholesterol	128.5 $\pm$ 16.4	131.4 $\pm$ 12.5
Campesterol	544.0 $\pm$ 15.0	567.1 $\pm$ 12.9
Stigmasterol	214.6 $\pm$ 8.4	222.0 $\pm$ 1.2
Sitosterol	420.2 $\pm$ 3.2	452.4 $\pm$ 6.5
Fucosterol	146.6 $\pm$ 10.3	158.5 $\pm$ 10.1
Δ5-Avenasterol	9.5 $\pm$ 0.7	10.6 $\pm$ 0.5
Cycloartenol	17.7 $\pm$ 3.0	3.5 $\pm$ 0.3
Total	1488.7 $\pm$ 49.5	1557.1 $\pm$ 38.5

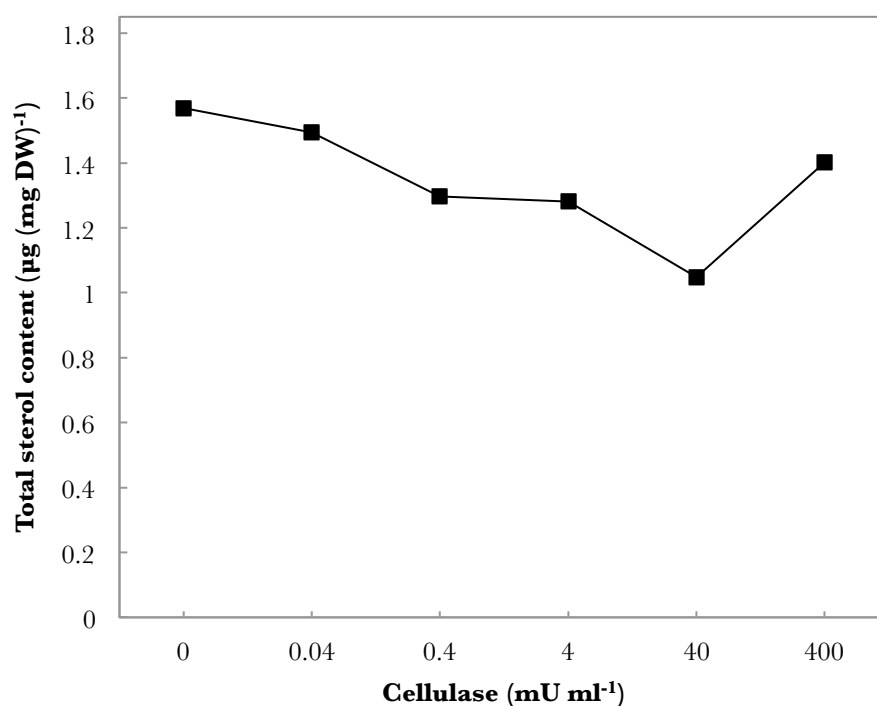


Figure 6.14. Effect of varying *T. viride* cellulase concentrations on BY-2 cell culture sterol concentrations.

T. viride cellulase was added to four-day-old cell suspension cultures. Cultures were harvested after 10 h under standard culturing conditions and total sterols were extracted. Data-points represent single measurements. No capsidiol was measured in chloroform extracts from these cultures. 4 U ml⁻¹ cellulase was also tested, but such a high concentration resulted in total cell lysis. This was confirmed by later replication of the application of 4 U ml⁻¹ cellulase. There were no visible differences between the remaining cultures, and FW was similar between cultures (mean $\pm$ standard deviation of 93.4 ± 1.3 mg ml⁻¹).

Table 6.3. Effect of 40 mU ml⁻¹ *T. viride* cellulase on BY-2 cell culture sterol content.

T. viride cellulase was added to four-day-old cell suspension cultures. Cultures were harvested after 10 h under standard culturing conditions and total sterols were extracted. Values indicate mean $\pm$ SEM measurements. There were no statistically significant differences in the concentration of individual or total sterols between the two conditions (student's *t* tests). No capsidiol was measured in chloroform extracts from these cultures.

	Sterol content (ng (mg DW) ⁻¹)	
	0 mU ml ⁻¹ cellulase	40 mU ml ⁻¹ cellulase
Cholesterol	8.7 $\pm$ 3.2	11.5 $\pm$ 0.8
24-methylene cholesterol	111.8 $\pm$ 13.3	101.7 $\pm$ 10.6
Campesterol	570.2 $\pm$ 40.5	559.3 $\pm$ 6.7
Stigmasterol	224.6 $\pm$ 19.3	207.8 $\pm$ 2
Sitosterol	470.2 $\pm$ 49.2	412.8 $\pm$ 5.9
Fucoesterol	138.7 $\pm$ 8.7	119.5 $\pm$ 8.8
Δ5-Avenasterol	11.8 $\pm$ 0.7	9.5 $\pm$ 0
Cycloartenol	12.3 $\pm$ 1.7	9.1 $\pm$ 0.2
Total	1548.4 $\pm$ 113.2	1431.4 $\pm$ 4.3

Table 6.4. Effect of 40 mU ml⁻¹ *T. viride* cellulase on BY-2 cell culture extractable microsomal HMGR activity.

Replicate cultures of those used for sterol and capsidiol analysis were subjected to microsomal extraction and HMGR assay. Values are means $\pm$ SEM of three replicates. Differences were not statistically significant (student's *t* test).

Treatment	HMG-CoA consumption (pmol min⁻¹ (mg protein)⁻¹)
0 mU ml⁻¹ cellulase	315 $\pm$ 17
40 mU ml⁻¹ cellulase	364 $\pm$ 40

6.3. Discussion

6.3.1. Transformation of tobacco BY-2 cells with a *P_{CaMV35S}-HMG1* construct led to increased sterol yields

BY-2 cell lines transformed with Arabidopsis *HMG1* under the control of the cauliflower mosaic virus 35S promoter had up to 1.8 times the control line sterol content. These increases were largely attributable to increases in sitosterol and, to a lesser extent, campesterol, while the pathway end product stigmasterol was unchanged. This is similar to the change in sterol profile observed in tobacco plants with increased HMGR activity (Gondet et al., 1992; Chappell et al., 1995; Schaller et al., 1995; Holmberg et al., 2003; Harker et al., 2003b), which makes for a persuasive argument that HMGR activity was successfully increased in *HMG1* lines, even in the absence of supportive enzyme assay data. However, in the absence of enzymological data, this cannot be unequivocally stated.

One difference between the sterol composition changes in *HMG1* over-expressing BY-2s and tobacco plants with increased HMGR activity is that the tobacco plants tended to have the greatest proportional increases in cycloartenol, whereas cycloartenol was rarely detected in transgenic BY-2 cells. This suggests that SMTI may exert less control over flux into sterols in WT BY-2 cells than in tobacco plants, in which over-expression of *SMT1* reduced the cycloartenol content of plants with increased HMGR activity (Holmberg et al., 2003).

6.3.2. Sterol yield increases were temporary

Within six months of initial sterol assays, sterol content and HMGR activity in *HMGI* lines were not significantly different to control levels. The heritability of increased sterol phenotypes had been observed to decrease progressively through generations in tomato plants over-expressing an HMGR gene, even though there were no obvious pleiotropic effects (Enfissi et al., 2005). This may be the case with the *HMGI* BY-2 lines, which did not show any obvious growth defects. However, *HMGI* cultures did appear less healthy by seven days after subculturing. Cell viability assays may have been able to deduce the extent of this, and metabolite assays could have provided evidence of cells being under stress. It should also be noted that growth assays were performed after sterol assays. It is, therefore, possible that the high sterol phenotypes were already lost by that time, although the lines appeared to be growing normally at the time of sterol assay.

It is possible that *HMGI* over-expression had subtle pleiotropic effects. Increased flux into sterols could have resulted in increased flux into brassinosteroids derived from campesterol, which are involved in regulation of cell division and differentiation (Clouse, 2011). Increases in BY-2 HMGR activity and cell death can be invoked by subjecting cells to farnesol, an analogue of FPP, and it may be the case that increased HMGR activity is mechanistically involved in cell death (Hemmerlin and Bach, 2000). Increased FPP synthase in *Arabidopsis* has also resulted in cell death (Masferrer et al., 2002), which implies that increased flux into FPP, rather than increased HMGR activity, might be responsible for cytotoxicity. However, BY-2 cells treated with farnesol could be rescued by addition of mevinolin, which inhibits HMGR (Hemmerlin and Bach, 2000), and the senescence observed in *Arabidopsis* was thought to be related to depletion of mevalonate leading to a decrease in cytokinins and the subsequent expression of *SAG12*, which encodes a cysteine protease (Masferrer et al., 2002). An alternative explanation is that the deregulation of mevalonate pathway flux in the

transgenic BY-2 cells led to the accumulation of toxic intermediates, which, in the case of IPP, DMAPP and FPP, has been found to be toxic in bacteria (Martin et al., 2003; Sivy et al., 2011).

These results suggest that tight regulation of HMGR activity and mevalonate pathway flux may be required for cell health. This contradicts successful attempts at increasing HMGR activity without obvious morphological effects in tobacco plants (Harker et al., 2003b). However, such regulation may be more crucial in a single cell system than in a multicellular system, due to less metabolic flexibility in the single cell system. Therefore, it is likely that the failure to achieve stable transgenic *HMG1* lines was due either to the accumulation of toxic intermediates or the redirection of flux into other non-sterol isoprenoids. It seems less likely that it is due specifically to the accumulation of large quantities of sterols, although, given the effect of sterols on membrane fluidity and permeability (Schuler et al., 1991; Hartmann, 2004), this explanation cannot be entirely disregarded.

6.3.3. Further attempts at increasing sterol content through genetic engineering of the mevalonate pathway were unsuccessful

Further attempts at transforming WT and *ATCS* lines with the *P_{CaMV35S}-HMG1* construct failed. The success of transformation with the *P_{CaMV35S}* control construct indicated that the transformation procedure was adequate, and it was verified that the agrobacteria used for transformation contained the pBI121-*HMG1* vector. In this case, the failure may be related to the reasons for transgene suppression in the initial *HMG1* lines. Transformation may have been successful, but transformed cells may not have been viable. Previous attempts to transform BY-2s with Arabidopsis *HMG1* have apparently worked with high efficiency but

failed to yield dividing cells (Hemmerlin, 1997, PhD thesis cited in Hemmerlin and Bach, 2000). However, it is somewhat perplexing that transformation with the *P_{CaMV35S}-HMG1* construct produced dozens of antibiotic-resistant colonies in one experiment, at least some of which had the expected high sterol phenotype, but did not produce any in several later attempts.

Attempts to increase sterol yields in tobacco leaves by transient expression of *HMG1* also failed. This is suspected to be due to the three-day period between transformation and harvest of leaves being insufficient for sterols to accumulate at high enough levels for a difference between genotypes to be detectable, although it is also possible that the lack of phenotype was due to a failure in transgene incorporation. HMGR assays could have been attempted on leaf samples in order to provide some verification of this. However, the specific aim was to increase mevalonate pathway flux, of which sterol levels were considered a better indicator than extractable HMGR activity.

The lack of transformants from *P_{UBQ10}* constructs (whether *HMG1*, *tHMG1* or *mCherry* control) was probably primarily due to the difficulty of screening transformed BY-2 cell lines with phosphinothricin. Concentrations of phosphinothricin below 10 $\mu\text{g ml}^{-1}$ were evidently too low to select sufficiently against untransformed cells, and concentrations greater than that might have been lethal even to transformed cells. Selection using 25 $\mu\text{g ml}^{-1}$ phosphinothricin has been reported to select for transformed BY-2s (van der Honing et al., 2010), but so too have various concentrations between 2 and 10 $\mu\text{g ml}^{-1}$ (Ali et al., 2003; Du and Poovaiah, 2004; Kadota et al., 2004). Indeed, in an examination of the effectiveness of a range of 1 to 10 $\mu\text{g ml}^{-1}$ phosphinothricin to select for transformed BY-2 cells, 2 $\mu\text{g ml}^{-1}$ was found to be most effective (Nakamura et al., 2010). It is, additionally, possible that the reason for failure of transformation with *HMG1* and *tHMG1* constructs was the same as for the *P_{CaMV35S}-HMG1*

failure, although the use of *P_{UBQ10}* was intended to make the presence of the heterologous *HMGR* less detrimental to the cells.

6.3.4. Attempts at chemically eliciting an increase in HMGR activity were unsuccessful

Attempts were made at eliciting an increase in BY-2 HMGR activity using cellulase from *T. viride*. 0.1 µg ml⁻¹ cellulase of an unknown activity reportedly gave the maximal response in HMGR activity and capsidiol yield in tobacco KY-14 cells (Chappell et al., 1991). Therefore, it was considered that the absence of a response, in either sterol or capsidiol content, in BY-2s subjected to 0.1 µg ml⁻¹ (0.4 mU ml⁻¹) cellulase could have been due to a difference between the specific activity of the enzyme used in these experiments and that used by Chappell et al. However, later results indicated that 0.01-100 µg ml⁻¹ (0.04-400 mU ml⁻¹) *T. viride* cellulase in BY-2 cultures led to the production of no more than 5.5 µg (g FW)⁻¹ or 2.2 µg (ml CCF)⁻¹ capsidiol, when taking into account the limit of detection by GC-MS, without affecting sterol yield. This compares to 41-71 µg (g FW)⁻¹ capsidiol resulting from elicitation of KY-14 cells with 0.05-1 µg ml⁻¹ cellulase (Chappell et al., 1991).

It was, therefore, concluded that *T. viride* cellulase had no effect on capsidiol synthesis, HMGR activity or sterol biosynthesis in BY-2 cells. There is some speculation that BY-2 cells are unable to synthesise capsidiol (personal communication referenced in Hemmerlin et al., 2004). My results certainly do not refute this. Despite that, other studies show that a crude cellulase extract from *T. viride* elicits volatile synthesis in several higher plants after application to exposed petioles (Piel et al., 1997) or to Madagascar periwinkle (*Catharanthus roseus*) cell cultures

(Garnier et al., 1996), apparently due to an increase in jasmonic acid. As such, it is surprising that no response was observed in BY-2 cells subjected to sub-lethal cellulase concentrations.

6.3.5. A different experimental approach might have avoided difficulties in increasing HMGR activity

Unlike work with intact plants (Chappell et al., 1995; Enfissi et al., 2005; Muñoz-Bertomeu et al., 2007), attempts at stably increasing HMGR activity in BY-2 cells by constitutive transgene expression proved unsuccessful. Use of an inducible promoter would have enabled establishment of transgenic lines before subjecting cells to potentially lethal accumulation of toxic intermediates and might have allowed more direct control over the degree of *HMG1* expression. Cell lines could then have been analysed for levels of toxic intermediates, and other enzymes that may have helped to alleviate the accumulation of these toxic intermediates could have been targeted. Prevention of a continuing build-up of toxic compounds would also have reduced the selection pressure against transformants and, consequently, reduced the likelihood of transgene silencing occurring.

The lack of success at modulating HMGR activity in BY-2 cells suggests that increasing the activity of this enzyme in this system is difficult to achieve without detriment to the cells. Given that this seems most likely to be due to insufficient direction of increased mevalonate flux into sterol biosynthesis, increasing the activity of FPP synthase (Kim et al., 2010) or squalene synthase (Seo et al., 2005) before targeting HMGR activity may create a route for the metabolism of excess prenyl diphosphates and ensure that increased sterol biosynthetic flux can occur without significant detriment to the system. Co-expression with sterol methyltransferase 1 may also be of benefit, although this seems less likely to affect sterol or

prenyl diphosphate levels significantly in the absence of manipulations more closely associated with prenyl diphosphates.

6.3.6. Conclusions

Transformation of WT BY-2 cells with an *HMG1* construct resulted in transgenic lines that accumulated high levels of sterols. However, failure to detect increased microsomal HMGR activity suggested that these lines were not genetically stable. This was probably due to the accumulation of toxic intermediates or other non-sterol isoprenoids that affect cell cycle regulation. Subsequent attempts to increased HMGR activity by *HMG1* over-expression failed, most likely for similar reasons. Attempts to chemically elicit an increase HMGR activity were similarly unsuccessful. Together, these results suggest a necessity for tight regulation of mevalonate pathway flux in BY-2 cells.

Chapter 7. General discussion

7.1. Summary of results.

After establishing appropriate analytical methods (Chapter 3), *CITRATE SYNTHASE 4* (*ATCS*; Chapter 4) and *3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE 1* (*HMG1*; Chapter 6), from *Arabidopsis* (*Arabidopsis thaliana*), were constitutively expressed in independent tobacco (*Nicotiana tabacum*) Bright Yellow 2 (BY-2) cell lines.

Overexpression of *ATCS* resulted in stable transgenic lines with up to 2.6 times the citrate synthase (CS) activity of controls and an altered sterol profile, with an accumulation of end-of-pathway sterols. The *ATCS* line with highest CS activity (line 6) had a slow growth phenotype, increased tricarboxylic acid (TCA) cycle fluxes (Chapter 5; including a more than 40% increase in flux through CS), and accumulated sterols at 1.2 times the control levels. No increase in flux through CS in the *ATCS* line with lower CS activity (line 16) was detected, and total sterol levels in this line were unchanged. This is thought to be due to mislocalisation of the *ATCS* protein.

Transformation of the wild-type (WT) with *HMG1* led to the accumulation of sterols at concentrations up to 1.8 times those of the control lines, but transgene expression was subsequently lost. Further attempts to increase 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity in WT and *ATCS* backgrounds, by genetic and chemical means, were unsuccessful. This lack of success in increasing HMGR activity could be the consequence of toxic prenyl phosphates accumulating when HMGR activity is increased.

7.2. Citrate synthase exerts significant control over cyclic tricarboxylic acid cycle flux in BY-2 cells

The increased TCA cycle flux in the transgenic *ATCS* 6 cell line with increased CS activity suggests that CS has a high flux control coefficient with respect to TCA cycle flux. Although a similar increase in TCA cycle flux was not seen in a second transgenic *ATCS* cell line, the *ATCS* protein is thought to have been mistargeted in this line, and therefore this line is not considered to disprove the hypothesis that CS exerts significant positive control over flux through the TCA cycle.

Control of primary metabolic flux, including in the TCA cycle, is generally shared amongst several enzymes (Raines, 2003; Geigenberger et al., 2004; Araújo et al., 2012). This means that individual enzymes do not exert substantial control over flux. Nevertheless, the work here shows a significant role of CS in the control of TCA cycle flux in BY-2 cells and contributes to the current understanding of the control of primary metabolic flux.

With respect to TCA cycle flux, the control imposed by CS is consistent with studies on tomato plants, in which a 50% reduction in CS activity led to a decrease in flux between citrate and α -ketoglutarate in illuminated leaves (Sienkiewicz-Porzucek et al., 2008). When ATP is available from alternative sources to the TCA cycle, such as from photosynthesis, non-cyclic flux modes seem to dominate (Hanning and Heldt, 1993; Schwender et al., 2006; Tcherkez et al., 2011). Thus, the decreased flux through CS in illuminated tomato leaves with reduced CS activity may not be indicative of reduced cyclic flux but rather suggests that the flux control imposed by CS extends beyond the traditional cyclic flux mode of the TCA cycle that is found in heterotrophic BY-2 cultures.

It is, however, necessary to make clear that the positive control exerted by CS activity on TCA cycle flux identified in BY-2 cells may not apply in all situations. It seems likely that CS imposes strong control over flux through CS, but the control of flux through other areas of the TCA cycle may be more complicated. Where a cyclic flux predominates, and the primary result is the generation of reducing power for ATP synthesis, such as in heterotrophic cell cultures (Williams et al., 2008; Masakapalli et al., 2010), CS may have a large impact on TCA cycle flux. However, different metabolic demands result in different TCA cycle flux modes (Sweetlove et al., 2010), and the degree of control imposed by individual enzymes over fluxes within the TCA cycle is likely to change with the changing metabolic demands. For instance, there is little cyclic flux in hypoxic conditions (Rocha et al., 2010), and it is unlikely that significant cyclic flux could be tolerated in these conditions due to insufficient oxidative phosphorylation and a subsequent lack of NAD^+ , which is required for isocitrate dehydrogenase, α -ketoglutarate dehydrogenase and malate dehydrogenase reactions to proceed in the forward direction. During hypoxia, increased flux through CS might be supported by a concomitant increase in pyruvate decarboxylase activity, but this is unlikely to result in increased cyclic flux.

Intriguingly, despite a reduction of flux from citrate to α -ketoglutarate in tomato leaves with reduced mitochondrial CS activity in the light, dark respiration, as measured by CO_2 efflux, in that system was increased (Sienkiewicz-Porzucek et al., 2008). This suggested that CS actually has a negative effect on cyclic TCA flux (Araújo et al., 2012). This interpretation is contradicted by my research on BY-2 cells and is counterintuitive. As already discussed in Chapter 5, measurement of CO_2 efflux may be a poor indicator of TCA cycle flux, and it seems unlikely that alteration of CS activity would affect TCA cycle flux in such contradictory ways in two different systems that each probably have cyclic flux predominating. Nevertheless, it could be the case that in tomato plants an increase in the production of citrate triggers a

down-regulation of flux within other parts of the TCA cycle, with the result that cyclic flux decreases.

The differences in TCA cycle flux modes and their potentially different methods of regulation raise an important issue for metabolic engineering. The process of identifying enzyme targets to manipulate in order to alter metabolic flux has to take into consideration the route of metabolic flux in the target system, and one should be aware of the different flux modes that are likely to be in operation in different tissues, developmental stages and environmental conditions. This highlights the complexity of performing successful manipulations in whole plants, and allows one of the benefits of using heterotrophic cell cultures to be reiterated, which is that they can be grown in conditions in which they have a more stable metabolism. Still, the suggestion of different enzymes exerting different levels of control over pathway flux based on the metabolic state of the cell does also raise intriguing possibilities for metabolic engineering. An enzyme could be identified that exerts significant control over the accumulation of a target metabolite in some tissues or developmental states but not in others. Constitutive manipulation of that enzyme could, therefore, cause the target metabolite to accumulate only in tissues or at times in which it is biotechnologically useful for it to accumulate. A corollary of this is that if a particular cell-type is to be targeted, prior metabolic flux analysis (MFA) is likely to be necessary to determine the route of primary metabolic flux and allow sensible targeting of specific enzymes. However, cell-type-specific MFA in multicellular systems is currently technologically challenging (Shachar-Hill, 2013).

Despite the likelihood of control over TCA cycle flux varying with the demands placed upon the cycle, there is evidence of CS exerting significant control over flux in several systems. Overexpression of CS reportedly led to increased citrate exudation from various plant roots (de la Fuente et al., 1997; Koyama et al., 2000; Anoop et al., 2003) including tobacco (de la Fuente et al., 1997) (although attempts to reproduce this result in tobacco failed (Delhaize et

al., 2001; Delhaize et al., 2003)). Also, mitochondrial CS gene expression in the green alga *Chlamydomonas reinhardtii* was negatively correlated with flux from acetyl-CoA into fatty acids and lipid, suggesting that CS positively controlled flux of acetyl-CoA into the TCA cycle (Deng et al., 2013). In this algal study, growth rate was increased in lines with increased CS activity. This was posited to be due to increased ATP availability caused by increased TCA cycle flux, unlike in BY-2 cells with increased TCA cycle flux, which had reduced growth rate that was attributed to lower carbon conversion efficiency. However, the algae were grown in continuous light and no flux analysis was performed, so a complete TCA cycle may not have been in operation, and the conclusion made with respect to TCA cycle flux may therefore be erroneous.

The contradictory results regarding tomato (Sienkiewicz-Porzucek et al., 2008) and algal (Deng et al., 2013) studies may be indicative of the complexity of TCA cycle regulation with respect to different flux modes and demands placed upon the cycle. It appears that aconitase (Carrari et al., 2003), α -ketoglutarate dehydrogenase (Araújo et al., 2008), fumarase (Nunes-Nesi et al., 2007) and malate dehydrogenase (Nunes-Nesi et al., 2005) (but not isocitrate dehydrogenase (Sulpice et al., 2010), succinyl-CoA ligase (Studart-Guimarães et al., 2007) or succinate dehydrogenase (Araújo et al., 2011)) also exert significant control over cyclic TCA flux. This high degree of control shown by many of the TCA cycle enzymes may be indicative of the biological need to specifically control flux into specific intermediates of the TCA cycle due to the differing roles of each as precursors to biomass components. Nevertheless, CS may be critical for increasing flux into cytosolic citrate and within the early part of the TCA cycle when non-cyclic flux modes are predominating.

7.3. Control of sterol biosynthesis in tobacco BY-2 cells lies predominantly downstream of mitochondrial citrate synthesis

Although flux through mitochondrial CS and levels of sterols were increased in *ATCS* line 6, there was not direct evidence of increased flux through cytosolic acetyl-CoA or an increased steady state concentration of cytosolic acetyl-CoA in this line. As such, the 18% increase in sterols in *ATCS* line 6 may not have been a direct metabolic effect of the increased TCA cycle flux. However, the prediction of significant isocitrate dehydrogenase (IDH) flux occurring outside of the conventional TCA cycle, by NADP⁺-dependent IDH, in this line could be an indication that citrate is exported from mitochondria at higher rates than in lines with normal TCA cycle flux, which would support the hypothesis that increased CS activity leads to a greater supply of precursors for mevalonate isoprenoid biosynthesis pathway. While the subcellular location of the increased NADP⁺-dependent IDH flux is unknown, the presence of cytosolic (Gálvez et al., 1996), peroxisomal (Corpas et al., 1999) and plastidial (Gálvez et al., 1994) NADP⁺-dependent IDH isoforms increases the likelihood of this flux occurring outside of the mitochondrion.

It should also be noted that, despite sterol levels being slightly increased as a biomass output in this line, there is insufficient evidence to conclude that flux into sterols in *ATCS* line 6 had increased. This is because there was assumed to be effectively no turnover of sterols in the model, which may be an unrealistic assumption. Such being the case, the increase in total sterol yield of *ATCS* line 6 could instead reflect a decrease in the turnover of sterols, although this does seem less likely than increased acetyl-CoA availability causing an increase in flux into sterols. Nevertheless, a decrease in brassinosteroid biosynthesis might result from decreased sterol turnover, and decreased brassinosteroid biosynthesis might produce a slow growth phenotype similar to that possessed by *ATCS* line 6 (Miyazawa et al., 2003).

A further point to consider is that very little of the increased TCA cycle flux in *ATCS* line 6 was channelled into sterols. Relative to a glucose uptake flux of 10, there was a difference of two flux units between flux through CS in *ATCS* line 6 and flux through CS in the control line with highest CS flux. By comparison the predicted difference in ATP-citrate lyase flux was only 0.01. Had all of the increase in CS flux been channelled into sterol biosynthesis, sterols levels would have increased by approximately 20 times. This suggests that CS actually exerts very little control of flux into sterols.

Given that an increase in CS activity caused a significant increase in the rate of mitochondrial citrate synthesis without greatly increasing sterol yields, and that sterols are derived from mitochondrial citrate, the control of flux into sterols must be primarily located downstream of citrate synthesis. This is consistent with the near doubling of sterol content achieved by overexpressing *HMG1* and the numerous papers that demonstrate considerable control of flux into sterols by enzymes of the mevalonate (Chappell et al., 1995; Schaller et al., 1995; Wang et al., 2012) and sterol biosynthetic pathways (Holmberg et al., 2002; Holmberg et al., 2003; Harker et al., 2003b; Seo et al., 2005).

7.4. Precursor supply from primary metabolism should be considered when engineering increased secondary metabolite yields

Previous attempts at increasing flux into specific secondary metabolites have caused pleiotropic effects by reducing the supply of metabolic precursors to competing branches of secondary metabolism (Hashimoto et al., 1993; Fray et al., 1995; Shewmaker et al., 1999; Richter et al., 2005; Fraser et al., 2007) or primary metabolism (Bohmert et al., 2000). My research instead shows that increasing the availability of a precursor from primary metabolism

can facilitate an increase in flux into a desired secondary metabolite. The degree of increased flux into sterols was small. Nevertheless, this and the previous research demonstrate the necessity of considering the wider metabolic network, including precursor supply and competing reactions, when altering metabolic flux by genetic engineering.

Other systems besides BY-2 cells may be more suitable for directly addressing the core issue of whether increasing the supply of primary metabolite precursors to specific secondary metabolic pathways can alleviate the negative effects of secondary pathway manipulations on the rest of metabolism. For instance, addressing the apparent depletion of plastidial acetyl-CoA in *Arabidopsis* expressing a heterologous pathway for polyhydroxybutyrate biosynthesis (Bohmert et al., 2000) may give more direct support for the hypothesis, although this would entail either using intact plants, creating a heterotrophic cell line from the plants, or successfully introducing a polyhydroxybutyrate biosynthetic operon into an existing heterotrophic cell line. Using intact plants with multiple cell types creates difficulties with respect to performing flux analysis and the multiple fluxomes in operation, while use of cell cultures may be infeasible given the severity of the phenotype in intact plants.

The high degree of control exerted by enzymes catalysing reactions within secondary metabolism on the accumulation of secondary metabolites (Yun et al., 1992; Chappell et al., 1995; Rosati et al., 2000; Estévez et al., 2001; Muir et al., 2001; Holmberg et al., 2002; Zhang et al., 2004) suggests that, in most cases, the supply of precursors to secondary metabolism is unlikely to significantly limit flux through native secondary metabolic pathways. Therefore, and as has been demonstrated for BY-2 sterol biosynthesis, increasing the supply of precursors is unlikely to produce large yield increases in secondary metabolism. In such a situation, manipulating the branch of metabolism leading from primary metabolism to the desired secondary metabolite is most likely to increase flux into the desired product. This appears to be most reliably achieved by targeting the first committed step at metabolic branch points

(Misawa et al., 1994; Fray et al., 1995; Rosati et al., 2000; Estévez et al., 2001; Zhang et al., 2004; Fraser et al., 2007). However, exceptions do occur, as indicated by the large degree of control over sterol biosynthesis exerted by HMGR, the third enzyme involved in the biosynthesis of isopentenyl diphosphate from acetyl-CoA.

Following the targeting of enzymes initially exerting a high degree of control over flux, steps initially exerting less control can be targeted progressively until the supply of precursors is addressed. This, unfortunately, entails a laborious process of performing serial transformations and screening transgenic lines after each transformation. Such an approach requires optimisation of expression levels to ensure that toxic intermediates do not accumulate, and manipulations often do not have the desired effect. While the method has been successful at increasing amorphadiene yields from *E. coli* (Martin et al., 2003; Pfleger et al., 2007; Pitera et al., 2007; Kizer et al., 2008), it is less desirable in plants due to the lengthier process of creating and analysing transformants.

Use of transcription factors may provide a more viable means of increasing flux into secondary metabolites, as these often target enzymes across the metabolic network, and there is evidence of the co-ordinated expression of genes encoding secondary pathway enzymes with genes involved in the supply of the pathway precursor from primary metabolism (van der Fits, 2000; Sønderby et al., 2007; Malitsky et al., 2008). This approach may be more difficult to achieve with novel pathways, but, if appropriate promoters are incorporated into transgenic DNA sequences, transcription factors could still be used to co-ordinately regulate enzyme levels and achieve high product yields.

Systems biology can be used to complement engineering approaches by determining logical genes for manipulation. Flux balance analysis (FBA) provides a viable means of determining gene candidates for overexpression as well as down-regulation. Enzyme targets can be found

by setting a high level of product formation as an objective function and using software to calculate which reactions have the greatest impact, either positively or negatively, on product formation (Ranganathan et al., 2010). Enzymes predicted by this method to influence flux into secondary metabolites have been manipulated to successfully increase secondary metabolite yields in bacteria (Hong et al., 2003; Choi et al., 2010; Xu et al., 2011b). Although plant research lags behind bacterial research, FBA models are now available for several different plant systems (Poolman et al., 2009; Hay and Schwender, 2011a), and these have been validated against experimental observations (Williams et al., 2010; Hay and Schwender, 2011b; Cheung et al., 2013).

Lastly, synthetic biology can be used to create mutations that are likely to favourably alter the post-translational regulation of the enzymes encoded by those genes. In addition to facilitating mutations that specifically affect enzyme regulation, synthetic biology may also be used to engineer protein scaffolds to allow substrate channelling. This can reduce the metabolic burden of synthesising enzymes, as lower enzyme concentrations are required to maintain a high metabolic flux. This has also been successfully implemented in bacteria (Dueber et al., 2009). However, synthetic biology approaches currently favour high-throughput analysis of multiple transgenic events and so may be ill suited to plant applications.

The work presented here demonstrates the relevance of precursor supply to secondary metabolite yield in plants, but the choice of enzyme to manipulate to increase precursor supply is critical. The high failure rate of transgene expression to give the desired effect on product yields when the selection of the target to manipulate is based solely on research in different organisms in different metabolic states suggests that greater emphasis should be put on considerations of the unique metabolic phenotype of the system that is to be modified. In the absence of appropriate data on the metabolic system in question, performing predictive

metabolic flux analysis prior to genetic engineering is likely to be the quickest and most reliable method of identifying enzyme targets for manipulation.

7.5. Conclusions

This work demonstrates that, although flux control in primary metabolism may be divided between many different enzymes, increases in the activity of single enzymes can nevertheless significantly alter flux, as revealed by the control exerted by CS over TCA cycle flux in tobacco BY-2 cells. However, TCA cycle flux may be controlled differently in different systems. Although CS probably contributes to the control of flux into sterols, the degree of this control is small. Control of flux into sterols instead lies primarily downstream of citrate synthesis, within the demand steps of the pathway rather than in the supply of the precursor. However, investigating this in cell cultures was hampered by pleiotropic effects resulting from manipulations to the activity of the enzyme presumed to exert most control over flux into sterols. A different engineering approach to the one used here may be required to elucidate this, but a different system may be more likely to demonstrate conclusively that manipulations to primary metabolism in plants can overcome the depletion of precursors to competing pathways that is caused by increasing flux through specific branches of secondary metabolism. Even so, this thesis demonstrates that the provision of precursors from primary metabolism can quantifiably affect the accumulation of secondary metabolites. Certainly primary metabolism and, specifically, the precursor supply should not be overlooked when engineering secondary metabolism, especially as the degree of control exerted by supply steps will increase as the activity of downstream enzymes is optimised (Fell, 1997).

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