

P5–GENERAL PLANT METABOLISM

Organised by M.M. Burrell for the Plant Metabolism Group. The Plant Metabolism Group gratefully acknowledge support from Advanced Technologies (Cambridge) Ltd.

P5.1–R1—A missing link in starch degradation?

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The network of starch degradation and its regulation is still not completely understood. Mutant plants selected for high starch content provide excellent tools to study starch metabolism. One of the most important mutants of this type is the *sex1* mutant of the model plant *Arabidopsis thaliana*. Mutants were identified by screening a chemically mutagenised population for high starch contents in leaves after prolonged periods of darkness and were therefore termed starch excess (*sex*) mutants (Caspar et al., 1991). The phenotype reveals that the *SEX1* protein is required both for starch degradation and for the phosphorylation of starch. The *sex1* mutant is defective in a protein known as R1 which was shown by a map based cloning approach (Yu et al., 2001). This starch associated protein has the activity of a starch:water dikinase (Ritte et al., 2002). However, its mode of action in the plant remains completely unknown. To understand precisely the role of R1 in this context will help us to get more insights in the tightly regulated network of starch degradation. Attempts to answer questions like what controls the pacing mechanism of starch breakdown or with what kinds of proteins does R1 interact will shed more light on the processes occurring in leaves in the dark.

Caspar et al. (1991) *Plant Physiol.* 95: 1181–1188

Ritte et al. (2002) *Proc Nat Acad Sci USA*, 99 (10): 7166–7171

Yu et al. (2001) *Plant Cell* 13 (8): 1907–1918

P5.2—The characterization of starch branching enzyme in wheat endosperm

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Starch is synthesized from sucrose imported via the phloem in storage organs such as developing wheat endosperm. ADPglucose is the immediate soluble substrate for starch biosynthesis. The enzymes which add glucose units from the nucleotide sugar to the growing polymer are all found within the amyloplasts. Granule bound starch synthase is responsible for the initial step in amylose synthesis, whereas the combined action of soluble starch synthases with the activity of starch branching enzymes (SBE) leads to amylopectin synthe-

sis. SBE catalyzes branch point formation in linear regions of starch. The introduction of branching into the growing starch polymer dramatically increases starch synthesis efficiency and is responsible for many of the physical and functional properties of starch.

To improve the functional properties of starch it is important to understand the roles and regulation of the specific isoforms of SBE. Using amyloplasts isolated from wheat endosperm we have purified three different isoforms of SBE by FPLC. Native polyacrylamide gel electrophoresis followed by activity staining confirmed the identity of these multiple isoforms. Furthermore, antibodies have also been used to distinguish between the different forms. The role and regulation of these SBE isoforms is now being further characterized.

P5.3—Modelling carbohydrate metabolism in stored potato tubers

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The experimental manipulation of plant metabolism, e.g. via generation of transgenic plant lines, often is very expensive and time-consuming. Therefore we are applying a more theoretical approach, i.e. modelling, which provides a framework for the planning and execution of experiments.

A problem of great economic interest is the search for transgenic lines of potato (*Solanum tuberosum*) with reduced or suppressed cold-sweetening (the accumulation of sucrose in the tubers during their storage at low temperatures). To assist this search, promising target enzymes for a genetic manipulation can be identified by the analysis of a model of the carbohydrate metabolism in stored potato tubers.

Here we present such a metabolic model. An analysis of the structure of the network, only requires stoichiometric information of all reactions involved. It was carried out with the software package ScrumPy.

By adding more information such as (i) metabolite concentrations, (ii) elasticities which characterize the kinetic properties of isolated reactions and (iii) information about the distribution of the carbon fluxes in the stored tubers, the model can be extended to allow metabolic control analysis (MCA) with emphasis on the control over sucrose concentration.

The results and implications of the structural analysis of our model and the issues involved in the extension of this model to a MCA model will be discussed.

P5.4—Single cell measurements of vacuolar hydrolases

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There are a variety of methods for characterising gene expression at the level of individual cells and for demonstrating that the cells also contain the encoded proteins. However, measuring the activity of enzymes at the resolution of single cells in complex tissues such as leaves is problematic. We have addressed this by using single-cell sampling to extract 30–100 pI-sized droplets of sap from individual plant cells and then measuring enzyme activities in these droplets with nanolitre-scale fluorescence-based assays. We have optimised these assays and used them to measure and characterise the activities of acid phosphatase and cysteine protease in sap samples from epidermal and mesophyll cells of barley (*Hordeum vulgare* L.) leaves undergoing artificial senescence. During leaf senescence we found that the dynamics with which acid phosphatase and protease activities changed were different in each cell type and did not mirror the changes occurring at the whole leaf level.

P5.5—Maltose metabolism in *Arabidopsis*

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Maltose is emerging as an important intermediate in starch synthesis and break down. We have identified two mutations (*mta1-1* and *mta1-2*) in a gene coding for a putative chloroplastic membrane protein. Our data indicate that this protein may be a novel maltose transporter that represents a crucial step in the degradation of leaf starch in *Arabidopsis thaliana*. In addition novel methods for the analysis of malto-oligosaccharides during starch synthesis will be discussed and results will be presented that indicate a role for maltose and longer malto-oligosaccharides during starch synthesis.

P5.6—The identity of photosynthetic compounds released from symbiotic algae to their animal hosts

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Dinoflagellate algae of the genus *Symbiodinium* in corals and related marine animals release much of their photosynthetically-fixed carbon to the animal. Algal cells freshly isolated from the symbiosis release low molecular weight compounds, e.g. glycerol, glucose and organic acids, but the relevance of these data to photosynthate release in the symbiosis is uncertain. To explore

the identity of the released compounds by *Symbiodinium* cells in symbiosis with the sea anemone *Anemonia viridis* symbiosis, we compared the pattern of metabolites in the animal tissue after incubation with, first, $^{14}\text{CO}_2$ under photosynthesising conditions and, second, a panel of ^{14}C -labelled organic substrates under non-photosynthesising conditions. In all experiments, the majority of the ^{14}C was TCA-soluble. After incubation with $^{14}\text{CO}_2$, ^{14}C was distributed between the neutral, acidic and basic fractions of the TCA-soluble compounds in the ratio 1:2:1. Prominent ^{14}C -labelled compounds included glucose, malate and two unidentified sugar phosphates. Incubation with single organic substrates (glucose, glycerol, citrate, fumarate, malate and succinate), resulted in the recovery of the majority of ^{14}C from only two of the three TCA-soluble fractions, but glucose and succinate supplied together gave a pattern of ^{14}C incorporation that closely matched that recorded with $^{14}\text{CO}_2$. These data suggest that glucose and succinate (or closely allied metabolites) may be the principal photosynthetic compounds transferred from the *Symbiodinium* cells to the animal tissues in *Anemonia viridis*.

P5.7—Genomic and functional analysis of two novel genes controlling mitochondrial and chloroplast development

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Mitochondria are ubiquitous and vital organelles found in all eukaryotes. Despite their critical importance, our understanding of the mechanisms that control their development is very limited, especially in higher plants. Previously, a number of stably inherited plant mitochondrial mutants have been isolated using EMS-mutagenesis of an *Arabidopsis thaliana* line with GFP-tagged mitochondria. These mutants exhibit a range of discreet morphological changes in mitochondrial size, shape, number and distribution; and may be used as a powerful tool for understanding the systems behind mitochondrial division and fusion in higher plants. Two of these mutants will be discussed. The *MMT1* mutant exhibits an increase in both mitochondrial and chloroplast size, and additionally increases the heterogeneity of size in both organelles. The *NMT1* mutant shows a constitutive change in mitochondrial morphology, from spherical, to an extended network of vermiform mitochondria. Positional cloning is being used to identify these mutant genes in preparation for analysis of their function. Data from these mutants will then be combined with information from other plant mitochondrial mutants in an attempt to create a robust model of mitochondrial development in higher plants.

P5.8—Sedoheptulose-1,7-bisphosphatase, an important enzyme for carbon assimilation and accumulation

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Sedoheptulose-1,7-bisphosphatase (SBPase) catalyses the dephosphorylation of sedoheptulose-1,7-bisphosphate, in the photosynthetic carbon reduction (Calvin) cycle. This reaction functions in the regenerative part of the Calvin cycle resulting in ribulose-1,5-bisphosphate, the CO₂ acceptor molecule. It has been shown previously using antisense technology, that SBPase has a high flux control coefficient, up to 0.75 over photosynthetic CO₂ assimilation. More recently it has been demonstrated that a small reduction (20%) in SBPase activity affects the allocation to sucrose and starch, growth rates and yield. These data suggest that it could be possible to increase carbon assimilation rates and plant growth by increasing SBPase activity in transgenic plants. Transgenic lines in which the wild type SBPase content has been increased by expression of an *Arabidopsis thaliana* SBPase cDNA. Analysis of these transgenic tobacco lines with increased levels of SBPase protein grown in low light and low temperature showed no differences in photosynthetic rate or yield. In contrast to these results, plants grown in high light, high temperature has revealed an increase in yield, leaf area and total mass of between 20–30% above the wild type. Increases in photosynthetic CO₂ assimilation at the plant level were also evident and changes in carbohydrates partitioning were observed.

P5.9—CP12, a novel plant protein involved in Calvin cycle regulation

S. Britliff and C. Raines (University of Essex)

Abstract not supplied

P5.10—A focus on the fascinating world of one-carbon metabolism

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The metabolism of one-carbon (C₁) units is vital to all organisms, and in plants is a relatively under investigated area of biochemistry. The network of pathways supplies C₁ units required for the synthesis of molecules such as proteins, pantothenate and an array of methylated species including S-methylmethionine. Despite the

low abundance and lability of many of the enzymes and intermediates of these reactions, pathway fluxes are high, particularly in plant species rich in methylated compounds such as lignin and betaines. Central to plant C₁ metabolism are the folate-dependent and independent pathways. The present project focuses on folate independent biochemistry, particularly the detoxification routes of methanol, formaldehyde and formate. Methanol and its metabolites arise from a number of sources including pectin demethylation and atmospheric pollution. The key genes in this pathway encoding: (1) glutathione-dependent formaldehyde dehydrogenase (FADH), (2) S-formylglutathione hydrolase (FGH) and (3) NAD-dependent formate dehydrogenase (FDH) have been identified and cloned in *Arabidopsis* and rice. FGH has also been cloned from wheat and overexpressed in *E.coli*, enabling characterisation of the purified protein. Northern analysis has been employed to investigate the transcript abundance of the three enzymes in *Arabidopsis* and rice following environmental and chemical stimuli. Antibodies have been raised to rice FDH and FGH enabling study of protein expression by western blotting. We have also used cDNA microarray approaches to examine patterns of gene expression in response to chemical stimulation. T-DNA insertion mutants for FGH and FDH have been identified, and future work will involve characterisation of these.

P5.11—Glucosinolate Biosynthesis: where primary and secondary metabolism overlap?

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Glucosinolates (GSLs) are plant secondary metabolites derived from a small number of amino acids. They occur in plants of the order Capparales, which includes several major crop species and the model plant *A. thaliana*. The GSL molecule consists of a common glycone moiety and a variable aglycone side chain. Over 100 different side chain structures occur, and about 50% of these are derived from methionine. Following tissue disruption, GSLs are hydrolyzed to an array of products of which isothiocyanates (ITCs) are the most prominent. ITCs are involved in mediating plant–herbivore interactions. Within vegetables, they are important flavour compounds, and may also have a role in reducing the risk of cancer. The synthesis of methionine-derived GSLs can be considered in three parts. Firstly the synthesis of chain elongated forms of methionine, secondly, the formation of methylthioalkyl GSLs, and lastly, various side chain modifications. Methionine chain elongation proceeds by the conversion of methionine to a keto acid, and then condensation with acetyl CoA, which acts as

the donor of additional methylene groups. The condensation reactions are catalysed by methylthioalkylmalate (MAM) synthases. The role of several MAM synthases in GSL biosynthesis has been confirmed in *A.thaliana*, though the precise pathway still remains unclear. In order to refine this model I am now investigating their role in *Brassica*. I will report the cloning of *Brassica* MAM synthases, their position within the *Brassica* genome in relation to QTLs which regulate glucosinolate biosynthesis, and their analysis via transformation and investigation of natural alleles.

P5.12–Lysine metabolism in cereal crops

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Cereal seeds are deficient in certain amino acids, in particular lysine. Biochemical, molecular and genetic studies have increased our knowledge concerning the regulation of the aspartate pathway, which synthesises lysine. Among the enzymes involved in lysine metabolism, aspartate kinase (AK) and dihydrodipicolinate synthase (DHDPS) control the regulation of lysine biosynthesis, whereas lysine: 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH), have been shown to play a key role in the breakdown of lysine. Lysine overproduction can be obtained by altering the sensitivity of DHDPS to lysine, but accumulation of this amino acid in cereal seeds requires further manipulation of LOR and/or SDH. These latter two enzymes have been shown to be specific to the endosperm tissue and their activities are drastically reduced in the high-lysine mutants of maize. We have isolated, purified and characterized the enzymes AK, HSDH, LOR and SDH from rice and coix seeds. The results indicate that the regulation of the LOR activity is complex and involves a calcium-dependent phosphorylation/dephosphorylation mechanism. We analysed the activity of the enzymes involved in lysine metabolism, characterized the storage proteins and determined the soluble lysine content for several opaque and floury maize mutants and show that only some of the mutants are similar to the well studied opaque-2 mutant. The results suggest that lysine accumulation in maize seeds cannot be explained by an increase in soluble lysine, but may be mainly due to an altered storage protein composition.

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P5.13–Metabolic signalling: Role of SnRK1 in regulating sterol biosynthesis

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SnRK1 is the plant homologue of yeast SNF1 and mammalian AMPK. It regulates carbon metabolism through the control of gene expression. For example, antisense expression of SnRK1 in potato has led to reduced sucrose synthase gene expression in tubers and loss of sucrose inducibility of sucrose synthase gene expression in leaves, indicating a requirement for SnRK1 in induction of sucrose synthase gene expression. SnRK1 also controls the activity of metabolic enzymes by phosphorylating them directly. So far, three important biosynthetic enzymes have been shown to be inactivated by SnRK1 phosphorylation, HMG-CoA reductase, sucrose phosphate synthase and nitrate reductase.

The ability to manipulate carbon metabolism and source/sink partitioning in crop plants could lead to improvements in yield and quality. We are attempting to use our knowledge of SnRK1's role in metabolic signalling to redirect carbon along specific metabolic pathways. In particular, to channel more carbon into the isoprenoid pathway by removing the control on HMG-CoA reductase exerted by SnRK1. We aim to determine whether such carbon redirection via manipulation of the SnRK1/HMG-CoA reductase interaction is feasible. Comparisons between SnRK1-controlled and uncoupled HMG-CoA reductase will also give insights into the metabolic and environmental signals that affect SnRK1 activity.

P5.14–The impact of 14-3-3 knock-outs on the nitrogen metabolism in *Arabidopsis*

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The eukaryotic class of 14-3-3 proteins is involved in the regulation of many biological processes such as membrane transport, primary and secondary metabolism, organelle targeting, and pathogen response. In plants, 14-3-3 proteins modify the activities of key enzymes of carbon and nitrogen metabolism such as sucrose-phosphate synthase, nitrate reductase and glutamine synthetase. The activity of these enzymes changes upon binding of 14-3-3 homo- or heterodimers to

specific phosphorylated recognition sequences within the target protein. In this context, it is tempting to suggest a crucial role of 14-3-3 proteins in the co-ordination of carbon and nitrogen metabolism with each member of the 14-3-3 family being involved in specific processes. However, the organization of 14-3-3 proteins in small gene families with high homologies between the individual members, their action as homo- and heterodimers and the ability of individual isoforms to bind to a great number of target proteins *in vitro* leads to the question of specificity vs. redundancy of these proteins. To further this discussion we chose a reversed genetic approach and isolated several T-DNA tagged 14-3-3 *Arabidopsis* mutants. Here, we present the metabolic analysis of a mutant disrupted in the 14-3-3 kappa gene.

P5.15–Phloem L-ascorbic acid: synthesis or transport?

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L-Ascorbic acid (vitamin C; L-AA) is a vital human nutrient obtained primarily through plant dietary sources. L-AA is ubiquitous in plants and is particularly abundant in green tissues where it plays a key role in photosynthetic metabolism. High levels of L-AA are also detected in storage organs (e.g. fruits, roots and tubers) of many plants. These organs constitute the main or unique nutritional component of many food crops and represent an important source of dietary vitamin C. The identification of the key determinants of L-AA accumulation in plant storage organs represents a primary target for the goal of enhancing the nutritional value of food crops.

Although the generality of plant tissues seem competent for L-AA synthesis, a recent report (Franceschi and Tarryn (2002) *Plant Physiol.* 130:649) provides evidence of long-distance transport from photosynthetic sources to sink tissues via the phloem. We investigated the role of phloem transport on L-AA distribution within the plant. Substantial levels of L-AA were detected in phloem exudates from a wide variety of plants. Surprisingly, phloem exudate from courgette fruits was found to contain significant *in vitro* activity for all soluble enzymes of the L-AA biosynthetic pathway. In addition, L-AA biosynthesis from unlabelled or ¹⁴C-labelled precursors in isolated celery vascular tissues was found to be higher than in adjacent parenchyma. Evidence for transport and phloem unloading of L-AA in storage organs was obtained in potato. The relative contribution of long-distance transport and phloem biosynthesis to L-AA accumulation in storage organs will be discussed.

P5.16–Assimilate partitioning in an *Arabidopsis* knockout mutant lacking the chloroplast triose-phosphate/phosphate translocator

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The chloroplast triose-phosphate/phosphate translocator (TPT) mediates the counter-exchange of stromal triose-phosphates derived from carbon dioxide fixation with cytosolic phosphate, thus providing the cytosol with precursors for sucrose synthesis and replenishing the pool of inorganic phosphate in the stroma. The *Arabidopsis* *tpt-1* mutant (identical to *ape-2*) in which the TPT gene is disrupted by a T-DNA insertion is phenotypically normal when grown in low light; in high light, however, photosynthesis is inhibited and growth is retarded relative to wildtype. Labelling studies using ¹⁴CO₂ establish that leaves of the mutant synthesise sucrose in the light, but only at 60–70% of the rate of wildtype. In low light the decreased flux to sucrose in the mutant is matched by increased labelling of starch, but in high light any increase in starch production is much less pronounced. Partitioning into other cellular components is largely unaffected at either growth irradiance. It is proposed that the mutant compensates for the absence of TPT by diverting photosynthate into starch, which is hydrolysed and exported from the chloroplast as glucose; the latter is phosphorylated by hexokinase in the cytosol to provide the substrates for sucrose synthesis; but while the capacity for phosphate release through starch synthesis is sufficient to allow normal rates of photosynthesis in low light, in high light it cannot fully compensate for the TPT defect. This explanation implies extensive turnover of starch in leaves of the *tpt-1* mutant in the light. Pulse-chase studies are being conducted to establish the extent to which this occurs.

P5.17–Metabolic signalling pathways involved in the regulation of carbon and nitrogen metabolism

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We seek to dissect the mechanisms involved in metabolite sensing and signalling and their role in the control of C and N partitioning in plants. We have focused on a protein kinase that plays a key role in the control of carbon metabolism. It was identified first in yeast and called sucrose non-fermenting 1 (SNF1); the plant equivalent is called SnRK1 (SNF1-Related Kinase-1).

Three enzymes, HMG-CoA reductase, sucrose phosphate synthase and nitrate reductase, act as substrates for SnRK1 *in vitro*. SnRK1 also regulates carbon metabolism through the control of gene expression. For example, the introduction of an antisense SnRK1 gene reduces sucrose synthase gene expression in potato tubers and α -amylase gene expression in cultured wheat embryos. Genetic manipulation of SnRK1 also affects plant development. For example, expression of antisense SnRK1 causes abnormal pollen development and male sterility in barley and inhibits tuber sprouting in potato. We have characterized several proteins that interact with SnRK1. These include SnIP1 (SnRK1-interacting protein-1), which has some similarity with the yeast SNF1 activator, SNF4, and an *Apetela2*/ethylene-responsive element binding protein-type transcription factor. More recently we have begun to study an *Arabidopsis* homologue of the yeast protein kinase, GCN2, which is required for cells to respond to amino acid availability. We have shown that *Arabidopsis* GCN2 will complement the *gcn2* mutation in yeast.

Zhang et al. (2001) *Plant Journal* 28, 431–442.

Slocombe et al. (2002) *Plant Molecular Biology* 49, 31–44.

Halford et al. (2003) *Journal of Experimental Botany* 54, 467–475.

P5.18—Characterisation of the glutathione-dependent formaldehyde detoxification pathway in *Arabidopsis*, wheat and rice

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Formaldehyde is a toxic compound that plant cells encounter as an atmospheric pollutant or as a product of endogenous de-methylating reactions. In order to prevent its accumulation to phytotoxic concentrations, formaldehyde is rapidly bound to tetrahydrofolate or chemically coupled to the endogenous nucleophile glutathione (GSH) to form S-hydroxymethylglutathione. S-hydroxymethylglutathione is subsequently oxidised by the action of a glutathione-NAD-dependent formaldehyde dehydrogenase (FMDH) to form the thioester S-formylglutathione. The activity of S-formylglutathione hydrolase (FGH) regenerates GSH and renders formate available to the C1 pathway. Alternatively, formate can be further metabolised to carbon dioxide by formate dehydrogenase (FDH). The latter represents the folate-independent pathway, which is the focus of our investigations. We have identified and cloned FGH from *Arabidopsis*, wheat and rice. Over-expression of recom-

binant FGH from wheat and rice showed a high level of activity. To clarify the key enzymes of the folate-independent pathway, fragments of FMDH and FDH were cloned from the three model species. This has allowed their transcript expression to be assessed in response to environmental and chemical stimuli using Northern analysis. In addition, characterisation of *Arabidopsis* T-DNA knock-out lines for the key enzymes FDH and FGH, identified from stocks held at the Salk Institute, is under way. It is apparent that the glutathione-dependent formaldehyde detoxification pathway is shared by species of wide phylogeny. It is therefore likely that in the course of evolution, this ancient biochemical pathway has become more important in the stress response of present-day organisms and less significant in their energetic metabolism.

P5.19—Cloning lipid dioxygenases involved in mushroom flavour

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Agaricus bisporus, commonly known as the button mushroom, has a world annual value of 4.5 billion Euro and is of commercial interest since it converts low or negative agricultural wastes (cereal straw, animal manure) into high value food. The success of this industry depends on its ability to deliver high quality mushrooms to the market. Quality is defined by colour, texture, firmness, maturity, and flavour.

Mushroom flavour is mainly due to C8 volatile compounds, and especially to the aliphatic alcohol 1-octen-3-ol, also called 'mushroom alcohol'. Octenol is derived from first the oxygenation and then the cleavage of a polyunsaturated fatty acid: linoleic acid. This is analogous to the cleavage of linoleic acid in plants resulting in oxo acids and volatile C6 and C9 aldehydes with their characteristic 'fresh green' odour. There are two possible types of enzymes to perform the reaction of oxygenation: lipoxygenases (non-heme dioxygenases), present in plants, fungi and mammals, or heme-dioxygenases, present in mammals (the cyclooxygenase family) and in fungi, with the recent discovery of the enzyme linoleate diol synthase in the fungus *Gaeumannomyces graminis*. Although both enzymes are candidates to catalyse the reaction, an examination of the enzymic mechanisms and fatty acid chemistry suggests that a heme-dioxygenase is involved in the production of 1-octen-3-ol.

Based on sequences of related genes, from plants and mammals, and on linoleate diol synthase gene sequences from fungi, degenerate primers have been designed in order to isolate genes involved in fatty acid metabolism from *A. bisporus* and *G. graminis*.

P5.20—Regulatory phosphorylation of phosphoenolpyruvate carboxykinase in plants

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Phosphoenolpyruvate carboxykinase (PEPCK) catalyses the ATP-dependent decarboxylation of oxaloacetate to phosphoenolpyruvate in plants. This is a key step in photosynthetic CO₂ concentrating mechanisms in some C₄ plants, Crassulacean Acid Metabolism plants (and algae). It also plays a key role in gluconeogenesis in C₃ plants. We are working with the C₄ plant, *Panicum maximum*, to investigate the regulatory phosphorylation of PEPCK. Previously, this group has shown PEPCK is phosphorylated and that the response of PEPCK to its substrates, PEP and OAA, depends strongly upon the adenylate ratio in the assay and changes with its phosphorylation state, resulting in its activation by dephosphorylation in the light and inactivation by phosphorylation in darkness. We have obtained the sequence for PEPCK from *P. maximum* by using RACE PCR and primers corresponding to highly conserved regions downstream of the N-terminus. The PEPCK gene in *P. maximum* is most similar to known C₄ PEPCK genes, particularly maize, but less similar to C₃ PEPCK genes. *P. maximum* PEPCK has one potential phosphorylation site which shares homology with sites recognised by cAMP-dependent protein kinases. We are confirming the phosphorylation site by ³²P labelling and sequencing of radiolabelled peptides after enzymatic digestion and purification of PEPCK. We have also made a construct of *P. maximum* PEPCK which has now been over-expressed in *E. coli*. We will modify the N-terminal sequence by site-directed mutagenesis and determine the effect of such manipulations on kinetic characteristics.

P5.21—Nitrogen Metabolism in *Canavalia ensiformes*

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Nitrogen is the most limiting essential nutrient for plant growth. Among nitrogen fixing microorganisms the *Rhizobium* group associate with legume plants. However, when nitrate is available in the environment it is preferred and is reduced to ammonium by the enzymes NR and NiR, being finally assimilated by the GS/GOGAST system. In tropical legume plants species, mainly those of the Phaseole tribe, when ammonium is produced by the symbiotic association the plant translocates preferably ureides via xylem, whereas when nitrate is the source

the plant translocates mainly amides such as asparagines and glutamine. The objective of this study was to identify in *Canavalia ensiformes* (L.), alterations in the sites and amounts of nitrogen and asparagine metabolism dependent upon the concentration of nitrate supplied to the plant from the early stage of germination to the reproductive stage. Different treatments had little effect on total soluble amino acids, protein and ureides at a particular developmental stage. The major changes occurred when different stages of development are compared, particularly with the beginning of the reproductive stage, observing a switch of the site of nitrate reduction, alterations in the total concentration of ureides and amino acids, when compared to the vegetative stage. Nitrate reductase activity of *Canavalia ensiformes* was shown to be regulated by the concentration of glutamine present in the tissues, exhibiting higher activity in tissues containing lower concentrations of glutamine, coinciding with the alteration of the site of nitrate reduction with the changing of the developing stage. (Financial support by FAPESP, Brazil)

P5.22—Soluble amino acid distribution in maize endosperm mutants

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For human nutrition the main source of vegetable proteins are cereal and legume seeds. The amino acid content of mature endosperm of wild-type, opaque and floury maize mutants were determined by HPLC. The total concentration of soluble amino acids varied significantly depending on the mutant. The o11 and 013 mutants exhibited the highest totals, whereas o10, f13 and f11 exhibited the lowest totals. In general, the mutants exhibited similar concentrations of total soluble amino acids when compared to the wild-type lines, with the clear exception of mutants o11 and f11. The o11 mutant exhibited a higher concentration of total soluble amino acids when compared to its wild-type counterpart W22 and the f11 mutant a lower concentration when compared to its wild-type counterpart Oh43. For methionine, the mutants o2 and o11 and wild-type Oh43 exhibited the highest concentrations of this amino acid. Significant differences were not observed between mutants for other amino acids such as lysine and threonine. The high lysine concentrations indicated originally for these mutants might be due to the amino acids incorporated into storage proteins, but not in the soluble

form. . (Financial support by FAPESP, Brazil, and the British Council).

P5.23—Analysis of enzymes involved in the biosynthesis and degradation of lysine in selected populations of maize

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Cereal seeds utilized for animal and human nutrition are deficient in essential amino acids such as lysine, threonine and tryptophan. Distinct strategies have been used to obtain high-lysine lines, which include induction and selection of biochemical mutants, production of transgenic plants with altered enzymes involved in lysine biosynthesis and long term breeding programs but only lysine concentration being characterized. Maize populations derived from the maize genetic bank of the University of São Paulo, Brazil, have been used in a breeding program designed to produce high-lysine maize lines. The activities of aspartate kinase (AK) and homoserine dehydrogenase (HSDH) involved in lysine and threonine biosynthesis, lysine 2-oxoglutarate reductase (LOR) and saccharopine dehydrogenase (SDH), both controlling lysine degradation in seeds, were tested in ten maize populations of the high-lysine breeding program. Results revealed a large variation in activities, particularly for LOR and SDH enzymes. In general, the activity of SDH was higher when compared to LOR. Three populations exhibited the highest LOR and SDH activities, indicating that the lysine concentrations were probably reduced in these genotypes. As expected, the QPM and o2 genotypes exhibited the lowest LOR and SDH activities confirming the lower rates of lysine degradation and as a consequence, lysine accumulation in the endosperm. The results suggest plant breeding may not reach the lysine contents that have been observed in the mutants and transgenic plants that have already been produced. However, the lines exhibit excellent agronomic characteristics, particularly yield, which normally correlates negatively with lysine accumulation. (Financial support by FAPESP, Brazil)

P5.24—Are polyamines involved in the resistance of barley to *Blumeria graminis*

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Polyamine levels and diamine oxidase and polyamine oxidase activities have been investigated in the first leaves of barley *Hordeum vulgare*, inoculated or uni-

noculated with conidia of powdery mildew (*Blumeria graminis* f. sp. *hordei*). Two cultivars, sensitive to powdery mildew viz. Chariot (resistant) and Golden Promise (susceptible) were used. The levels of putrescine, spermidine and spermine were found to be higher in the leaves of Chariot than in the leaves of Golden Promise and, with the exception of spermine, were generally higher in both cultivars after inoculation. In inoculated leaves of Chariot, putrescine levels and diamine oxidase and polyamine oxidase activities increased to a maximum at 9 days post inoculation and spermidine levels were maximal at 12 days. In controls (uninoculated leaves), the activities of these enzymes and putrescine and spermidine levels also increased but not to the same extent as in inoculated leaves. With Golden Promise, the levels of putrescine and spermidine in the inoculated leaves changed very little over sampling times but were always higher than in the controls. In this cultivar, there was little difference between inoculated leaves and the controls in diamine oxidase activity which reached a maximum value at 9 days post inoculation. Activity of the bound form of diamine oxidase was low in both the cultivars. Polyamine oxidase was not detected at 3 days after inoculation in either cultivar but activity at fairly low levels was recorded at later times, usually reaching a maximum value at 9 days. The results suggest that polyamine metabolism and diamine oxidase activity in particular may be involved in the mechanism conferring resistance to barley powdery mildew in Chariot.

P5.25—The fructose bisphosphate aldolase multigene family in *Arabidopsis thaliana*

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Class I aldolase is found in all animal and higher plant tissues. Fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) catalyses the reversible reaction of fructose 1,6-bisphosphate into D-glyceraldehyde 3-phosphate and dihydroacetone phosphate via aldol cleavage or condensation. Aldolase is required in the glycolytic/gluconeogenic pathways and the Calvin cycle in plants and is known to be compartmentalised within cells. Little is known about the regulation of aldolase; however, the activity and distribution of isoenzymes are known to be regulated by both environmental conditions and/or during plant development. A search of the *Arabidopsis* genome database has revealed 3 plastidic and 5 cytosolic putative isoforms. Investigation of their mRNA expression patterns will result in a greater understanding of the role of aldolase (and multigene families). A plastidic aldolase has been purified from *Brassica oleracea* leaves and used to raise a polyclonal antibody. Western blot analysis using this and a cytosolic aldolase antibody has

been used to examine expression of these proteins in cucumber cotyledons and leek and also the abundance in different tissues of *Arabidopsis*. Seven primer pairs have been designed for the 8 isoforms of aldolase in *Arabidopsis* (2 genes are tandem repeats with >95% similarity). Four pairs have been used to study the mRNA expression in *Arabidopsis* flower development. Data will be presented to show that the abundance of isoenzymes is altered during development and by environmental conditions and that individual isoenzymes show different tissue-specific expression.

P5.26–Ceramide biosynthesis, uncovering its role in plant biology

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Ceramide is a unique lipid compound in that it consists of two acyl-chains linked via an amide bond. Ceramide is synthesized by the enzyme ceramide synthase from a C18 acyl-amide, otherwise known as a long chain base, and a C16 to C24, CoA activated, fatty acid. The resulting ceramide may be glycosylated with a variety of compounds, including glucose, galactose, mannose, and inositol phosphate, to produce a bewildering complexity of lipids each with specific functions in membrane biology and protein chemistry. Ceramide biosynthesis is essential for normal growth of plant cells, but interestingly also seems to be involved in their death. Disruption of ceramide biosynthesis by the ceramide synthase inhibitor AAL-toxin, results in programmed cell death in certain sensitive genotypes. This is associated with increases in the amount of free long chain bases and a decrease in certain ceramide-containing lipids. This, and other evidence, indicates that long chain bases and ceramide are bioactive compounds. As a result their synthesis and degradation must be tightly controlled. However, if they are to function effectively as signaling molecules, both synthesis and degradation must be able to respond to and integrate external signals. We are investigating how *Arabidopsis thaliana* responds to changes in flux through the ceramide biosynthesis pathway using a variety of inhibitors and molecular genetic techniques.

P5.27–Effect of sugars on the regulation of senescence in *Arabidopsis thaliana*

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During leaf senescence, photosynthetic proteins are being degraded, which leads to the remobilisation of nitrogen in the plant. It has been shown that sugars are important signals in the regulation of a large range of

metabolic and developmental processes. In particular, it is likely that sugar accumulation can trigger the initiation of leaf senescence, especially in plants growing under nitrogen deficiency.

In this work, we are analysing the effect of sugars on the regulation of leaf senescence and on resource allocation in wild-type plants of *Arabidopsis* and in mutants with decreased sugar sensitivity. Plants were grown on four different media, high nitrogen (30 mM nitrogen) and low nitrogen (4.7 mM nitrogen) with or without 2% glucose. Using chlorophyll fluorescence imaging, we monitored the senescence-dependent decline in photosynthetic function. Senescence was accelerated in plants grown on medium with a combination of low nitrogen and 2% glucose. This observation was coupled with a decrease in total protein and chlorophyll contents. These results indicate interaction of the nitrogen and sugar signalling pathways during the regulation of senescence. *Arabidopsis* mutants showing sugar-insensitivity during early seedling development exhibited the same response as wild-type plants, suggesting that distinct sugar signalling mechanisms are involved in the regulation of early and late developmental processes. We are currently isolating *Arabidopsis* mutants with altered sugar sensitivity of leaf senescence.

P5.28–Characterisation of starch phosphorylase from developing wheat endosperm amyloplasts

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Starch phosphorylase catalyses the reversible transfer of glucosyl units from glucose-1-phosphate to the non-reducing end of alpha-1,4-linked glucan chains. As such it has the ability to both synthesise and degrade starch. In higher plants two major isozymes of starch phosphorylase exist (Pho1 and Pho2) that differ in subunit size, kinetic properties and intracellular localisation. The larger of the two isozymes, Pho1, has a low affinity for branched polyglucans such as glycogen and is exclusively plastidial. Pho2, on the other hand, has a very high affinity for branched polyglucans and is located solely in the cytosol. To provide an insight into the role of starch phosphorylase in starch biosynthesis, work has focussed specifically on the Pho1 isozyme located in the amyloplasts of the developing wheat endosperm; amyloplasts being the organelle responsible for the synthesis of starch in this tissue.

Pho1 synthetic and degradative activity, in amyloplasts isolated from developing wheat endosperms, has been demonstrated. Future analysis of the pattern of Pho1

activity throughout endosperm development will provide an understanding of the relationship between the activity of this isozyme and starch synthesis. In addition, a starch phosphorylase cDNA fragment, generated using RT-PCR, has been used to isolate putative full-length cDNA clones of Pho1 from an available cDNA library. Once the identity of these clones is confirmed, they will be used to characterise Pho1 gene expression to provide further information relating to Pho1 and its possible role in starch synthesis.

P5.29—Lipo-oligosaccharide induces root development in *Podophyllum hexandrum*

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Podophyllum hexandrum is an endangered Asian medicinal plant, but there has been limited success in establishing in vitro shoot cultures as a basis for efficient propagation systems. This is attributable, in part, to the short seasonal availability of this species and, crucially, difficulties in adventitious rooting of its shoots. To address the latter, this study has investigated, for the first time, the effectiveness of lipo-oligosaccharide, NGR234, a nod-factor produced by *Rhizobium* strain NGR234, in stimulating rooting of somatic embryo-derived plants of *P. hexandrum*. Somatic embryos were produced from embryogenic cell suspension cultures established from root-derived callus, cultured in liquid UM medium in the dark. Differentiation of somatic embryos and subsequent shoot formation were achieved when embryos were transferred to MS medium with 0.45 mg l⁻¹ benzylaminopurine. Somatic embryo-derived shoots lacking roots were cultured on half-strength MS medium, lacking growth regulators, but supplemented with 10⁵ NGR234 in the dark for 7 d at 22±1 °C. Plants were maintained under a 16 h photoperiod (42 µmol m⁻² s⁻¹) at 22±1 °C for 28 d. Rooting of >50% of plants occurred within 35 d but controls did not root. Overall, supplementation of culture medium with lipo-oligosaccharides is a novel approach for rooting somatic embryo-derived plants of *P. hexandrum* providing a realistic basis for its routine micropropagation.

P5.30—Metabolic profiling of transgenic lettuce

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Metabolic profiling of transgenic lettuce (*Lactuca sativa* L. cv. Evola) plants expressing an *ipt* gene under control

of the senescence-specific SAG12 promoter from *Arabidopsis thaliana* (*P*_{SAG12-*ipt*}) has been achieved using direct Atmospheric Pressure Chemical Ionisation Mass Spectrometry (APCI-MS) and Linked Gas-chromatography (GC) APCI/EI-MS techniques. Mature heads of transgenic lettuce and their azygous controls were maintained under defined conditions to assess shelf-life. In addition to significantly delayed senescence, head tissue derived from transgenic plants exhibited significant increases in the concentrations of four volatile organic compounds (VOCs), corresponding to a molecular mass of 45, 47, 51 and 63 (up to 3-fold, 3-fold, 2-fold and 3-fold higher, respectively) as compared to head tissue derived from azygous plants. The VOCs were identified as an acid aldehyde (acetaldehyde) (45), ethanol (47), methanol + protonated water (H₃O) (51) and dimethyl sulfide (63). Accumulation of methanol and dimethyl sulfide was concomitant with the accumulation of reactive oxygen species in transgenic lettuce heads. These results demonstrate the usefulness of applying metabolic profiling techniques to elucidating the underlying mechanisms determining physiological response of plants to transgene expression.

P5.31—Investigating the occurrence, metabolism and significance of CA1P and the branch chain precursors of CA1P

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Recent work from our laboratory has established a biosynthetic pathway for the Rubisco inhibitor, 2-carboxyarabinitol 1-phosphate (CA1P) from CO₂ via FBP, hamamelose phosphates, hamamelose and carboxyarabinitol [1]. This may explain why plants with increased amounts of chloroplastic FBP (achieved by altering the abundance of chloroplast FBPase [2]) also contained more CA1P and more of the associated intermediates [1]. The amount of CA1P never exceeded the Rubisco active site concentration, suggesting that unbound CA1P was metabolised, even at night. Light induction of photosynthesis by these plants became increasingly retarded as the amount of chloroplast FBPase diminished. In transformants with intermediate amounts of FBPase, this correlated with the amount of CA1P present rather than with the regeneration of RuBP, demonstrating the potential of CA1P to influence the onset of photosynthesis. We are currently attempting to identify genes associated with the metabolism of hamamelose in Arabidopsis. [1] Andralojc, P.J., Keys, A.J., Kossmann, J. & Parry, M.A.J. (2002) Proc. Natl. Acad. Sci. USA **99**, 4742–4747. [2] Kossmann, J., Sonnwald, U. & Willmitzer, L. (1994) Plant J. **6**, 637–650.

P5.32—Characterisation of *Chlorella pyrenoidosa* L-ascorbic acid accumulating mutants: Identification of an enhanced biosynthetic enzyme activity and cloning of the putative gene from *Arabidopsis thaliana*

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We have undertaken a comparative study of L-ascorbic acid (L-AA) biosynthesis in wild-type *Chlorella pyrenoidosa* and two mutant strains containing enhanced L-AA content. Both mutant strains synthesized L-AA more efficiently from the distant precursors D-glucose or D-mannose whilst enhanced biosynthesis was not observed from the immediate precursors L-galactose or L-galactono-1,4-lactone. In vitro assay of individual biosynthetic steps revealed that only one enzyme exhibited enhanced activity in both mutant strains, a putative GDP-L-galactose pyrophosphatase. Both mutant strains were found to contain higher concentrations of free L-galactose than the wild-type strain suggesting that GDP-L-galactose pyrophosphatase and/or L-galactose-1-phosphate phosphatase activity were also enhanced in vivo.

In order to simplify cloning of the pyrophosphatase gene, we chose to purify the enzyme from *Arabidopsis thaliana* due to the availability of the genome sequence. The activity was partially purified and a number of proteins were N-terminal sequenced by Edman degradation, one of which corresponded to a putative nucleotide pyrophosphatase-like protein. Nucleotide pyrophosphatase protein concentrations correlated well with enzyme activity as judged by Coomassie staining on polyacrylamide gels. Current efforts are focused on in vitro characterization of the recombinant enzyme and in vivo analysis of protein function.

P5.33—Switching from starch to lipid degradation during early pea seedling development

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Pea (*Pisum sativum*) is a starchy seed that also contains about 1.4% oil. The embryo utilizes these reserves during germination and early seedling development and we have observed that there is a switch in which food reserve is utilized at different stages of development. The first five days of development are not light-dependent. The seed becomes fully imbibed, then the radicle emerges followed by the plumule. Starch reserves are

utilized to fuel this initial growth and starch levels in the cotyledon decrease by up to 50% whilst lipid levels remain constant. Supplied carnitine does not affect this utilization of starch. Following emergence of the plumule, development is light-requiring and involves greening and massive development of the leaf tissues to produce a photosynthetic, autonomous plant. During this stage of development, lipid stores in the cotyledon are utilized whilst starch levels remain constant. Supplied carnitine doubles the rate of lipid utilized. This switch to lipid utilization coincides with the previously observed peak of mitochondrial beta-oxidation activity in the cotyledon (Masterson & Wood, 2001).

The attainment of autonomy in the leaves requires the biosynthesis of plastid pigments and the structural lipids and proteins of the chloroplast lamellae. Mitochondrial beta-oxidation in the cotyledons, which is carnitine-dependent, might provide metabolites needed for this and explain the stimulation of greening by carnitine previously observed (Wood et al., 2002).

Masterson & Wood 2001 Proc. R. Soc. Lond. B, 268, 1949–1953.

Wood et al. 2002 Comp. Biochem. Phys. A, 132 (Supplement 1), S178.

P5.34—Cell-specific nitrate storage and remobilisation in barley leaves

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Nitrate is an important N storage form in plants and is accumulated in the vacuole when uptake exceeds immediate metabolic requirements. We have investigated the role of different cell types in nitrate storage in barley leaves using single-cell sampling. A microcapillary was used to remove 50–100 pl droplets of vacuolar sap from individual cell types in the third leaf of barley plants and these were quantitatively analysed for nitrate and nitrate reductase (NR) activity using fluorescence-based microassays. When plants were grown with adequate nitrate from germination, both epidermal and mesophyll cells accumulated nitrate to maximum concentrations of about 250 mM, and there were no significant differences between cell types. However, if nitrate was not supplied until the third leaf had fully expanded, the nitrate concentrations were much lower being about 120 mM in mesophyll cells and 50 mM in epidermal cells. Detailed studies of the dynamics of nitrate accumulation over the first 48 h after supply of nitrate to fully-expanded leaves showed that increases in nitrate concentrations occurred mainly during the night and declined during the day. These patterns were related to the diurnal changes in NR activity and hence the balance between assimilation and storage. This was confirmed by comparing accumulation patterns in NR mutants and wild-type barley. When nitrate was removed from nitrate-replete barley plants, there was differential remobilisation of nitrate from epi-

dermal and mesophyll cells, with utilisation of the epidermal pool preceding that in the mesophyll by several days.

P5.35—A method for rapid profiling of metabolites in plant extracts

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With advancements in plant functional genomics there is a requirement for complementary methods to facilitate rapid comparison of metabolites, notably for the demonstration of substantial equivalence in transgenic crops. Non-targeted metabolite profiling is a valuable tool for comparing levels of chemically diverse compounds, allowing detection of unexpected changes which may be missed using directed metabolite assays, thereby enabling further understanding of pathway interactions.

We have developed a LC-MS-based technique to provide semi-quantitative information on metabolite levels in plant samples. Extracts are infused into the MS as a continuous stream via the LC and spectra are collected over a 3 minute period. Compounds are identified from their molecular mass, accurate to within 3 mDa. The method can in theory be applied to any ionisable metabolite, with minimal sample preparation and no requirement for derivitisation.

We have profiled aqueous metabolites from mature green fruit pericarp of a cultivated tomato (*L. esculentum*) and lines carrying defined introgressions from *L. pennellii* (Eshed and Zamir (1995)). A wide range of metabolites was detected in the extracts, including amino acids, organic acids and carbohydrates. Details of method verification and optimisation will be presented and data obtained from the introgression lines will be discussed.

Y. Eshed and D. Zamir (1995) *Genetics* 141, 1147–1162.

P5.36—Factors affecting carbon flow in the TCA cycle in isolated plant mitochondria

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The generation and utilisation of the mitochondrial proton electrochemical gradient, and the supply of key metabolic intermediates for biosynthesis, are the two principal functions of mitochondrial metabolism in heterotrophic plant tissue. Maintaining the correct balance between these functions requires flexibility in the under-

lying fluxes through the reactions of the TCA cycle, and much of the information about this process has been inferred from respiration rate measurements on isolated mitochondria. Information on the rate of ATP synthesis and carbon flow through the TCA cycle can be obtained directly by using nuclear magnetic resonance (NMR) spectroscopy to probe mitochondrial metabolism in defined respiratory states. This approach, in conjunction with oxygen electrode measurements, has been used to investigate factors affecting carbon flow in mitochondria isolated from potato (*Solanum tuberosum* cv. Desiree) tubers. Pyruvate was found to support very similar rates of ATP synthesis and oxygen consumption in the presence of citrate, malate or succinate (all at 10 mM), or under simulated cytosolic conditions, but NMR revealed differences in the metabolic fate of the carbon derived from pyruvate. These differences can be interpreted in terms of the metabolic transformations occurring in the mitochondria and the activity of the transporters that link the NMR-detectable pools in the suspending medium with the undetectable pools in the mitochondrial matrix. These experiments emphasise the flexibility of the metabolic network and its ability to meet a demand for ATP synthesis with different metabolic fluxes. Similar experiments can be used to highlight the consequences of inhibition and uncoupling.

P5.37—Utilization of glycine as a nitrogen source by plant roots

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Dissolved organic nitrogen provides an alternative to the usual inorganic nitrogen forms in the soil in a wide range of plants and circumstances. Glycine has been shown to be a significant source of plant nitrogen in some cases and it might be expected that the subsequent metabolism of the amino acid would be strongly dependent on root aminotransferase activity. However it is becoming apparent that the glycine decarboxylase complex (GDC) is an essential component of glycine catabolism in non-photosynthetic tissues and so it is necessary to characterise the metabolism of glycine by plant roots. The metabolic fate of the carbon and nitrogen derived from exogenous glycine by maize (*Zea mays* L.) root tips has been followed using in vivo NMR spectroscopy. The results show that the combination of GDC and serine hydroxymethyltransferase is essential for the metabolism of glycine in this heterotrophic tissue. At the same time the NMR experiments provide no evidence for the

involvement of the serine: glyoxylate aminotransferase that would have been expected to be largely responsible for glycine metabolism. Glycine has been shown to be an important component of dissolved organic nitrogen in a range of extreme habitats and it is possible that

external stress factors, such as salt or osmotic stress, influence the utilisation of glycine by roots. Sodium chloride at 100 mM is sufficient to affect the growth of maize seedlings and the effect of this stress on glycine utilization is being investigated.