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P3–CO₂ CONCENTRATING MECHANISMS

Organised by R.C. Leegood, A.M. Borland and K. Maxwell for the Plant Metabolism Group and for the Plant Environmental Physiology Group

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P3.1–CO₂ concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution

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Cyanobacteria have evolved an extremely effective single-cell CO₂ concentrating mechanism (CCM). Recent molecular, biochemical and physiological studies have significantly extended our knowledge about the genes and protein components of this system and how they operate to elevate CO₂ around Rubisco during photosynthesis. The CCM components include at least 4 modes of active inorganic carbon uptake, including two bicarbonate transporters and two CO₂ uptake systems associated with the operation of specialised NDH-1 complexes. All these uptake systems serve to accumulate HCO₃⁻ in the cytosol of the cell, which is subsequently used by the Rubisco-containing carboxysome protein microcompartment within the cell to elevate CO₂ around Rubisco. A specialised carbonic anhydrase is also present in this compartment. The recent availability of at least 7 cyanobacterial genomes has made it possible to begin to undertake comparative genomics of the CCM in cyanobacteria. Initial analyses (Badger et al., 2002) have revealed a number of surprising findings. Firstly, cyanobacteria have evolved two types of carboxysomes, correlated with the form of Rubisco present (Form 1A and 1B). Secondly, the two HCO₃⁻ and CO₂ transport systems are distributed variably, with some cyanobacteria (*Prochlorococcus marinus* species) appearing to lack CO₂ uptake systems entirely. Finally, there are multiple carbonic anhydrases, but surprisingly the marine *Prochlorococcus* and *Synechococcus* species appear to lack any identifiable CA genes. A pathway for the evolution of CCM components will be presented.

Reference:

Badger MR, Hanson DT, Price GD (2002) Evolution and diversity of CO₂ concentrating mechanisms in cyanobacteria. *Functional Plant Biology* 29: in press.

P3.2–The ecological meaning of CO₂ concentrating mechanisms in lichens

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Lichens are generally regarded as one of the most outstanding examples of symbiosis between algae (phycobiont) and/or cyanobacteria (cyanobiont) with a fungal partner (mycobiont). Associations with phycobiont are unique in that they can reactivate photosynthesis just with water vapor, whereas cyanobiont lichens require liquid water. Together with bryophytes, they share a *poikilohydric nature*, meaning that the water balance depends on the water conditions of the surrounding environment, meaning that CO₂ uptake may be transient, a function of thallus water content. Recently, it has been suggested that the activity of a CCM overruled the limitations to CO₂ fixation imposed by large diffusion resistances. Using several techniques based on carbon isotope discrimination and gas exchange, the CCM activity was observed in cyanobiont and in some phycobiont lichens, depending on the presence of a chloroplast pyrenoid. For cyanobacteria, lichenisation leads to the most pronounced CCM characteristics, with lowest values of isotope discrimination commensurate with both lower Rubisco fractionation and high DIC pools, low K_{1/2} for CO₂ and high carboxylation conductances. For phycobiont lichens with a pyrenoid, the lower CCM activity leaves a clear organic and instantaneous discrimination signal which is intermediate between cyanobiont and non-CCM phycobiont associations, as are DIC pools and other gas exchange characteristics. Finally, the analysis of lichen water use efficiency (LWUE), suggested that both water storage and gas exchange characteristics were maximized in cyanobiont species due to the operation of a CCM, indicating 'different ecological strategies'.

P3.3–To concentrate or ventilate: are there any advantages of a biophysical CCM for hornworts as compared to liverworts?

H. Griffiths, D. Richardson, K. Maxwell and W.E. Robe, Plant Sciences, Cambridge

The Anthocerotae (hornworts) are the one family of terrestrial plants to possess a pyrenoid-based CO₂ concentrating mechanism. The phylogenetic relationships between hornworts, liverworts and mosses has been the subject of some debate, and we have developed a comparative approach from a physiological perspective to infer how photosynthetic limitations might have acted as selective pressures during the colonisation of the land. The effect of the CCM on photosynthetic gas exchange and electron transport rates in *Phaeoceros* is contrasted with liverworts with a varying degree of thallus ventilation. When compared to the thalloid, non-ventilated *Pellia*, a considerable advantage is conferred by the CCM, but this disappears in the those liverworts with increasing internal air spaces, such as *Marchantia* and *Conocephalum*. We relate this to the likely rates of photorespiration, both currently and in palaeohistorical times. The occurrence of the CCM is associated with uniplastidic cells and we explore the implications for the development of the multiplastidic condition, loss of the pyrenoid and potential increase in mesophyll conductance in well-ventilated tissues.

P3.4—CO₂-concentrating consequences in crassulacean acid metabolism

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The consequences of CO₂-concentrating in leaf air-species of CAM plants during Phase III of CAM (=Crassulacean acid metabolism) are explored. There are mechanistic consequences of internal CO₂ partial pressures. These are (i) effects on stomata, i.e. high internal CO₂ partial pressures eliciting stomatal closure in Phase III, (ii) regulation of malic acid remobilization from the vacuole, malate decarboxylation and refixation of CO₂ via Rubisco (ribulosebisphosphate carboxylase/oxygenase), and (iii) internal signaling functions during transitions between Phases II and III and III and IV, respectively, in the natural day/night cycle and in synchronizing the circadian clocks of individual leaf cells or leaf patches in free running endogenous rhythmicity of CAM. There are ecophysiological consequences. Obvious beneficial ecophysiological consequences are (i) CO₂-acquisition, (ii) increased water-use-efficiency, (iii) suppressed photorespiration, and (iv) reduced oxidative stress by overenergization of the photosynthetic apparatus. However, a general potency of these beneficial effects may be questioned. There are also adverse ecophysiological consequences. These are (i) energetics, (ii) pH effects and (iii) Phase III oxidative stress. A major consequence of CO₂-concentrating in Phase III is O₂-concentrating, increased internal CO₂ partial pressures is accompanied by increased internal O₂ partial pressure. Do reversible shifts of C₃/CAM-intermediate plants between C₃-CAM-C₃ modes of photosynthesis tell us that C₃-photosynthesis provides the better protec-

tion from irradiance stress? There are many open questions and CAM remains a curiosity.

P3.5—C₄ photosynthesis—principles of CO₂ concentration and prospects for its introduction into C₃ plants

R.C. Leegood, Animal and Plant Sciences, University of Sheffield

C₄ photosynthesis has a number of distinct properties that enable the capture of CO₂ and its concentration in the vicinity of Rubisco, so as to reduce the oxygenase activity of Rubisco, and hence the rate of photorespiration. I will discuss the properties of this CO₂-concentrating mechanism, and indicate the minimum requirements of any genetically-engineered system. In particular, the Kranz leaf anatomy of C₄ photosynthesis and the division of the C₄-cycle between two cell types involves intercellular co-operation that requires modifications in regulation and transport to make C₄ photosynthesis work. Some examples of these modifications will be discussed. Comparisons will be made with the C₄-type photosynthesis found in single-celled C₄-type CO₂-concentrating mechanisms, such as that found in the aquatic plant, *Hydrilla verticillata* and the single-celled C₄ system found in the terrestrial chenopod *Borszczowia aralocaspica*.

P3.6—Crassulacean acid metabolism (CAM) is an adaptation to drought and light stress; fact or misinterpretation?

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Early studies on CAM were performed with species from arid habitats. The results let ecophysiologicals propose that CAM is an adaptation to desert environments. The finding that this putative adaptation to xeric habitats was present in aquatic plants questioned the widely accepted opinion resulting in a modified description. CAM serves as a CO₂ harvesting or retaining mechanism e.g. in aquatic plants. In terrestrial and epiphytic plants the advantage of CAM focus on its water conserving properties. Comparative investigations of CAM and C₃ plants showed that this generalisation may not be valid in all cases. Reduced water availability may reduce CAM. C₃ plants but not CAM species survived prolonged period without rainfall in the Namib desert. In sympatric species of one genus it could not be verified that CAM provide a superior performance compared to C₃ photosynthesis during drought. Drought may shift photosynthesis from exclusive C₃-pattern to 'typical' CAM. Drought also induces the CAM enzymatic machinery. CAM-plants respond to declining soil water by an increased accumulation of organic acid. Comparative studies indicate a niche preference for xeric habi-

tats in CAM—relative to C_3 taxa within a genus. These conflicting results challenge a re-evaluation of the ecological relevance of CAM. Special emphasis is given to the effects CAM probably has on photosynthesis under drought.

P3.7—The operation and significance of the CAM CCM in relation to leaf succulence

K. Maxwell, H. Griffiths and G. Lanigan, Plant Sciences, Cambridge

Members of the genus *Kalanchoe* exhibit extreme flexibility in the expression of CAM over the diel course, which has repercussions for carbon gain and light use. In general terms, thick-leaved more succulent species (e.g. *K. fedtschenkoi*) are more dependent on nocturnal CO_2 fixation than thinner-leaved species such as *K. pinnata*, which show a greater degree of plasticity in the expression of the CAM Phases. CAM imposes phase-dependent energetic requirements relating to the activity different temporal activity of PEPC and Rubisco. Diurnal patterns of photosynthetic electron transport differed markedly between species, depending on the extent of atmospheric CO_2 uptake mediated by Rubisco in the light. Further evidence that the more succulent CAM species are tightly locked into the CAM cycle is provided from data charting the recovery of linear electron flux over the nocturnal period. Electron transport is increasingly repressed over the course of the night and recovery following a period of light acclimation is strongly species-dependent. The data are related to the temporal separation of the C_3 and C_4 carboxylases as a function of the commitment to CAM made by the more succulent species.

P3.8—Carbohydrate partitioning in CAM plants: reconciling potential conflicts of interest

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Carbohydrate turnover is a central component in determining the magnitude of expression of CAM but also represents a potential constraint to the growth and productivity of CAM species. Multiple levels of control are likely to be required for switching the flux of carbon between the potentially competing sinks of CAM, respiration and export for growth over the course of the diel cycle and in response to longer-term changes in environmental conditions and shifts in development. The construction of diel leaf carbon budgets, together with analyses of the isotopic composition of biochemical fractions can be used to examine how CAM plants adjust carbohydrate partitioning in response to long-term shifts in sink demands. Carbohydrates also have impor-

tant implications in the short-term for controlling flux through PEPC over the 24 h CAM cycle. Since metabolites, probably cytosolic malate, have been shown to override the circadian control/activation of PEPC over the CAM cycle, we hypothesised that the reciprocating carbohydrates might also play a role in modulating PEPC kinase expression. Genes encoding β -amylase and starch phosphorylase, two enzymes implicated in starch degradation, show parallel diel expression in *Mesembryanthemum crystallinum*. The patterns of gene expression are consistent with patterns of enzyme activity/starch degradation and maximal levels of expression precede the up-regulation of PEPC kinase. Manipulating starch content via changes in environmental conditions and internal CO_2 supply indicates that circadian regulation of genes encoding starch degrading enzymes could ensure precise control of carbohydrate breakdown which, in turn, determines flux through PEPC.

P3.9—Transport processes in CAM

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Crassulacean acid metabolism (CAM) can be regarded, in essence, as a single-cell phenomenon. The metabolic events that characterize the temporal, bipartite separation of carbon flow during the day–night cycle all take place within the confines of individual photosynthetic cells. But by the same token, regulation of the two distinct phases of CO_2 fixation requires tight co-ordination of metabolite fluxes and enzyme activities. The central vacuole in CAM plants plays a crucial role in the day–night cycle of carbon fixation and assimilation. Nocturnally synthesized malic acid must be effectively transported out of the cytoplasm and sequestered in the vacuole. With the onset of the light period, the situation is reversed and there is large-scale efflux of malic acid out of the vacuole, matched by an equivalent rate of malate decarboxylation in the cytoplasm. Futile cycling across the vacuolar membrane must be minimized to avoid reducing the energetic efficiency of the CAM cycle, emphasizing the need for strict regulation of fluxes across the vacuolar membrane. This presentation will examine our current understanding of vacuolar transport processes in CAM plants. Considerable evidence suggests that malic-acid accumulation is brought about by an inward-rectifying malate-selective ion channel operating in parallel with the tonoplast H^+ pumps. Efflux appears to occur by a kinetically distinct transport system, most probably a proton-linked malate symport mechanism. Current investigations are aimed at revealing the ways in which these transport systems are regulated in synchrony with the underlying rhythm of CAM.

P3.10—Regulation of CAM PEP carboxylase

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Phosphoenolpyruvate (PEP) carboxylase catalyses the primary assimilation of CO₂ in Crassulacean acid metabolism (CAM) plants. It is activated by phosphorylation, which reduces its sensitivity to the allosteric inhibitor L-malate. This plays a major role in setting the day/night pattern of metabolism in these plants. The key factor that controls the phosphorylation state and in vivo activity of PEP carboxylase is the activity of PEP carboxylase kinase. Recent work on CAM plants has established this enzyme as a novel protein kinase and has provided new insights into the regulation of protein phosphorylation. PEP carboxylase kinase comprises a protein kinase catalytic domain with essentially no extensions that could contribute regulatory properties. It is controlled by synthesis/degradation in response to a circadian oscillator; both kinase message and kinase protein turns over rapidly. This circadian control of PEP carboxylase kinase can be over-ridden by changes in metabolite levels. The primary effect of the circadian oscillator in this system may be at the level of the tonoplast, and changes in the expression of the kinase may be secondary to circadian changes in the concentration of a metabolite, perhaps cytosolic malate. CAM plants also contain a protein that inhibits PEP carboxylase kinase. The role of this inhibitor protein may be to block the basal level of kinase activity that remains after completion of the acidification phase of CAM.

P3.11—Imaging and imagining the complex spatio-temporal dynamics of photosynthesis on simple leaves

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Crassulacean acid metabolism (CAM), which is regarded to be a time-dependent adaptation with a distinct day/night pattern of CO₂ exchange, is expressed as dynamic patterns of independently initiated spatial variations in photosynthetic efficiency over a single leaf. Spatio-temporal heterogeneity of photosynthetic efficiency (ϕ_{PSII}) governs the subsequent phases of CAM and the different periods of endogenous, circadian rhythm in continuous light. Non-invasive, highly sensitive chlorophyll fluorescence imaging reveals randomly initiated patches of varying ϕ_{PSII} which are propagated within minutes to hours in wave fronts, forming dynamically expanding and contracting clusters and clearly dephased regions of ϕ_{PSII} . These phenomena are now described for the obligatory CAM-plant *Kalanchoë daigremontiana* and for the facultative CAM-plant *Clusia minor*. Heterogeneity only emerges during CAM and disappears during C₃ photosynthesis, thus the spatial pattern formation can be considered to be related to the metabolic constraints of CAM with its substantial differences of leaf-internal CO₂ concentration. Tightly

packed mesophyll cells limit CO₂ diffusion inside CAM leaves and isolate adjacent leaf areas. Different leaf anatomy qualitatively influences the type of observed patterns, however, the variations are not simply related to leaf anatomy. Correlation analyses and mathematical quantification methods which are based on cellular automaton techniques, help to understand these heterogeneity of photosynthetic efficiency. Thus, the biological CAM-rhythm has to be considered as a spatio-temporal product of many individual oscillators on a single leaf, defined by CAM photosynthesis and by limitations of CO₂ diffusion between tightly packed cells.

P3.12—Does the underlying circadian oscillator which controls the daily CAM cycle have a molecular basis in the nucleus? Evidence from the model CAM plant *Mesembryanthemum crystallinum*

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The molecular mechanism controlling the circadian rhythm of CO₂ fixation in CAM plants is not understood. There has been a debate as to whether the daily movement of malate into and out of the vacuole during the CAM cycle could itself be the underlying circadian oscillator which controls the observed rhythms of CO₂ fixation. We have studied the regulation of over 40 genes in C₃ and CAM leaves of *Mesembryanthemum* under free-running conditions (LL). This study has revealed that many genes encoding enzymes of the CAM pathway are specifically controlled by the circadian clock in CAM leaves of *Mesembryanthemum*. Overall some 35 new clock-controlled genes have been identified in CAM *Mesembryanthemum*. We have identified the *Mesembryanthemum* homologues of several clock genes, which were originally characterized in *Arabidopsis*. We have determined that TOC1 and LHY/CCA1 are under reciprocal circadian control in a similar manner to their regulation in the *Arabidopsis* clock, such that TOC1 peaks around subjective dusk and LHY/CCA1 peak around subjective dawn. The regulation of TOC1 and LHY/CCA1 is not affected by CAM induction. We have found that homologues of FKF, ZTL and ELF3 are under circadian control in C₃ and CAM leaves. Whilst the transcript abundance of ZTL has not been found to oscillate in *Arabidopsis*, it does oscillate in *Mesembryanthemum* and most clearly in CAM tissues. Our observations will be integrated into a working model forming the basis for future experiments aimed at understanding the molecular events underlying the circadian control of CO₂ fixation in CAM plants.

P3.13–The use of genetic modification to understand the regulation of CAM cycles

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Plant genetic modification has proved to be a powerful tool in studying the regulation of metabolic pathways in C_3 plants. However, there has been little progress in applying this technology to C_4 and CAM plants. Here we describe the results of research where three transgenes have been used to study the regulation of fluxes of the CAM cycle in *Kalanchoë* plants. In the first series of experiments the amounts of fructose 2,6-bisphosphate in leaves of *Kalanchoë daigremontiana* were raised through the introduction of a rat liver gene that could synthesise fructose 2,6-bisphosphate. In these experiments it is shown that fructose 2,6-bisphosphate levels can control sucrose and starch synthesis during photosynthesis. Plants possessing raised amounts of fructose 2,6-bisphosphate displayed reduced rates of sucrose accumulation during photosynthesis and marked rises in starch accumulation. This observation was identical to earlier observations in transgenic tobacco plants containing the same transgene. However, the elevated fructose 2,6-bisphosphate amounts were not observed to influence the metabolism of malic acid reserves in the CAM cycle. Attempts to generate *Kalanchoë* plants possessing reduced amounts of fructose 2,6-bisphosphate using a second transgene were unsuccessful. In a third series of experiments the activity of the alternative oxidase was altered through the introduction of a sense construct of the Voodoo lily (*Sauromatum gutatum*) alternative oxidase gene. This produced plants possessing both elevated and reduced amounts of the alternative oxidase protein. Preliminary work suggests that these plants display marked differences in the CAM cycle.

P3.14–Genetic and molecular genetic dissection of CAM in the common ice plant

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Crassulacean acid metabolism (CAM), an important adaptation of photosynthetic carbon fixation to limited water or CO_2 availability, is present in approximately 7% of vascular plant species. Key features of CAM include nocturnal CO_2 fixation into C_4 acids by phosphoenolpyruvate carboxylase (PEPC), daytime CO_2 re-assimilation into carbohydrates by Rubisco, and improved water use efficiency. In contrast to C_3 and C_4 plants, which have well-developed genetic models, there has been no investment in the development of a genetic model for CAM. To overcome this limitation, we have established large mutant collections in the com-

mon ice plant, *Mesembryanthemum crystallinum*, as well as a facile screening method for the identification of CAM-defective mutants. Investigations of selected CAM-defective mutants are underway to define the underlying mechanisms that control the regulation of CAM induction by environmental stress and endogenous circadian rhythms (Cushman, 2001; Cushman and Borland, 2002). We have also established a *M. crystallinum* expressed sequence tag (EST) database and cDNA microarrays as starting points for functional genomic analyses to develop a detailed picture of the molecular genetic events that govern CAM induction and circadian regulation. In addition, the development of an efficient, non-tissue culture based ice plant transformation system will be necessary for the systematic functional analysis of the structural and regulatory requirements of CAM.

References:

Cushman JC (2001) Crassulacean Acid metabolism (CAM): A plastic photosynthetic adaptation to arid environments. *Plant Physiol.* 127: 1439–1448.

Cushman JC, Borland AM (2002) Induction of Crassulacean acid metabolism by water limitation. *Plant Cell Environ.* In press.

P3.15–Environmental consequences of the contrasting pattern of Rubisco use in C_3 and C_4 Plants

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The capacity of Rubisco to consume RuBP is a major limitation on net CO_2 assimilation rate (A) in C_3 and C_4 plants. The pattern of Rubisco limitation differs between the two photosynthetic types, as shown by comparisons of temperature and CO_2 responses of A and Rubisco activity. In C_3 species, Rubisco capacity is the primary limitation on A at light saturation and CO_2 concentrations below the current atmospheric value, particularly near the temperature optimum. Below 20 °C, C_3 photosynthesis at 37 and 68 Pa is often limited by the capacity to regenerate phosphate. In C_4 plants, Rubisco capacity is equivalent to A below 18 °C, but exceeds photosynthetic capacity above 25 °C, indicating Rubisco is an important limitation at cool but not warm temperatures. A Rubisco limitation on A in C_4 plants reflects their altered pattern of Rubisco use. C_4 plants express approximately 30% of the Rubisco as equivalent C_3 species. Because C_4 Rubisco functions near V_{max} in the CO_2 -enriched environment of the bundle sheath, it shows a very pronounced temperature response that leads to very low capacity at cool temperature, and excessively high capacity at high temperature. At low temperature, the low Rubisco capacity in C_4 plants causes the strong Rubisco limitation, leading in turn to low photosynthetic potential at low temperature relative to C_3 species. This may be the leading mechanism restricting C_4 plant performance in cool environments, and may

explain in part the rarity of C_4 plants at high altitude and latitude.

P3.17—Molecular evolution of C_4 photosynthesis—a case study with the genus *Flaveria*

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The enzymes of the C_4 photosynthetic pathway have evolved several times independently from C_3 ancestral enzymes. The C_3 isoforms have different metabolic functions and their kinetic and regulatory properties differ from those of the C_4 counterparts. In addition, C_4 and C_3 isoform genes display their own specific expression patterns. To unravel the key changes, which the C_4 genes have undergone for their role in C_4 photosynthesis, the PEP carboxylase (PEPC) gene family of the genus *Flaveria* (Asteraceae) was chosen as a model system. *Flaveria* is unique, because this genus contains C_3 and C_4 plants as well as a large number of C_3 – C_4 intermediate species, which are considered to be evolutionary intermediates towards C_4 photosynthesis. We have cloned the C_4 PEPC coding sequence from the C_4 species *F. trinervia* (gene designation: *ppcA*) and, in addition, the orthologous *ppcA* sequences from C_3 and C_3 – C_4 intermediate species of *Flaveria*. Biochemical and mutational analyses of the recombinant enzymes allowed us to identify determinants for the Km-PEP of both the activated and non-activated enzymes, and to propose a scenario for the evolution of the C_4 PEPC. In parallel to the analysis of the PEPC enzyme we have investigated the evolution of the *cis*-regulatory determinants for the mesophyll-specific expression of the C_4 *ppcA* gene. Using transgenic approaches a *cis*-acting element has been located in the upstream region of the *ppcA* promoter of *F. trinervia* that explains the quantitative and qualitative expression characteristics of this C_4 gene.

P3.18—Expression analysis of glycine decarboxylase in transgenic C_3 and C_3 – C_4 intermediate plants

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To gain further understanding of the cell-specific expression of glycine decarboxylase (GDC) in C_3 – C_4 photosynthetic intermediates, the model species *Moricandia arvensis* (Brassicaceae) was used. Most C_3 – C_4 intermediate species that have been studied to date express the four heterologous GDC subunits, the P, H, T and L proteins in a bundle sheath-specific manner. However, the C_3 – C_4 intermediate species *M. arvensis* shows a

unique expression pattern of the GDC subunits by expressing only the P protein of the enzyme complex bundle sheath-specifically and the H, L and T proteins in both mesophyll and bundle sheath cells (Morgan et al., 1993, *Planta* 190:468–473). In C_3 plant species, all GDC subunits are expressed in both cell types. In order to perform GDC promoter studies, an *Agrobacterium*-mediated transformation system was developed for *M. arvensis* by using the green fluorescent protein as a vital screenable marker. To gain insights into transgene behavior in the species *M. arvensis*, up to 100 independent plant lines were characterised at structural and expression level. To analyse GDC promoter directed expression in C_3 and C_3 – C_4 intermediate plants, the GDC-H and P promoter from *M. arvensis* and the GDC-P promoter from the C_3 species *Moricandia moricandioides* were fused to the β -glucuronidase (*gusA*-intron) gene and used for transformation of *M. arvensis* and the C_3 species *Nicotiana benthamiana*. In comparison to constitutive reporter gene expression, the activity of the GDC promoters and the expression patterns of their reporter gene fusions in these plant species will be discussed.

P3.19—Genetic manipulation of glycine decarboxylase

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Glycine decarboxylase (GDC) is a key component of the glycine–serine inter-conversion in all organisms. It comprises four different protein components and is metabolically linked to serine hydroxymethyltransferase. In plants, GDC plays a crucial role during photorespiration and represents the molecular source of photorespiratory carbon dioxide. Mutants defective in GDC have been described by Somerville and Ogren already approximately twenty years ago (*Biochem. J.*, 202: 373–380, 1982). Such mutations, as obtained by chemical mutagenesis in *Arabidopsis thaliana*, are lethal under conditions of aerobic photosynthesis. We have produced potato and *A. thaliana* plants impaired in the biosynthesis of GDC subunits. Transgenic potato plants with reduced levels of the glycine decarboxylating GDC subunit, P-protein, were obtained by an antisense approach. T-DNA insertion lines of *Arabidopsis thaliana* defective in each of the two individual genes encoding P-protein in this plant were isolated from appropriate mutant seed pools by conventional PCR screenings. In addition, some features of the old GDC mutant described by Somerville and Ogren have been re-investigated. Data from a comprehensive physiological and biochemical analysis of these plants will be presented and discussed in the light of possible contributions of GDC to the build-up of carbon dioxide concentration gradients within the leaf.

P3.20—Biochemical and physiological studies on transgenic maize overexpressing a sorghum C₄ phosphoenolpyruvate carboxylase

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The effect of modulating the amount of phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) on the C₄ photosynthesis pathway and the lipid and protein filling of seeds was investigated in transgenic maize. These plants were engineered by *Agrobacterium*-mediated infection or biolistic techniques with plasmids containing a full length C₄ PEPC (from sorghum) cDNA under the control of the C₄ gene promoter (leaf specific), or the HMWG promoter (seed specific). Seeds from the transgenic plants showed a 10-fold increase in PEPC activity, however, the impact on lipid and protein content was not clearly detected. Transformed maize overexpressing (B6-F1.1; 2.2 fold) or underexpressing (A6-F1.1; 0.2 fold) C₄ PEPC in leaves were characterised by Southern and Northern analyses. PEPC was expressed in the mesophyll cell cytosol and found to undergo phosphorylation at light. Under normal growth conditions, B6-F1.3 exhibited a low (7%) but statistically significant improvement in CO₂ assimilation rate (A) and no change in the intrinsic water use efficiency (WUE) while the down regulated A6-F1.1 plants showed both a markedly reduced A (−36%) and WUE (−42%). Under moderate drought WUE (+30%) and dry weight (+20%) increased in B6-F1.3, while the stomatal density (−22%) decreased. An opposite variation was seen for A6-F1.1 (A, −38%; WUE, −47%). In conclusion, emphasis should be put on (1) the remarkable plasticity and adaptive capacity of maize illustrated by the pleiotropic response to the genetic modification and (2) the improved photosynthetic performance of the transgenic plants—notably a better water use and dry matter production—under drought conditions.

P3.21—Overexpression of C₄ cycle enzymes in C₃ plants: A biotechnological approach to improve C₃ photosynthesis in transgenic potato and tobacco plants

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The process of photorespiration diminishes the efficiency of CO₂ assimilation and yield of C₃-crops. In an attempt to minimise photorespiration by a genetically introduced CO₂ concentrating mechanism, enzymes typical for the C₄-cycle were overexpressed in potato and

tobacco plants either individually or in combination. Overexpression of PEPC from the prokaryote *Corynebacterium glutamicum* in potato plants resulted in a pleiotropic increase in respiratory carbon loss in the dark and light accompanied by a slight decrease in the CO₂ compensation point (Γ^*). The increase in respiratory CO₂ release was most likely a consequence of a six-fold induction of the endogenous cytosolic NADP malic enzyme (ME). Moreover, potato transformants constitutively overexpressing an engineered version of potato PEPC (with a higher affinity for PEP and a decreased sensitivity towards malate inhibition) exhibited additionally stunted growth, accumulation of malate and amino acids combined with decreased rates of starch accumulation in the leaves. A combined overexpression of PEPC and chloroplastic NADP-ME alleviated the temperature response of CO₂ assimilation in air and resulted in a lower electron requirement for CO₂ assimilation with increasing temperature suggesting an attenuation of photorespiration. Interestingly, the induction of the endogenous cytosolic NADP-ME was absent in the double transformants, despite of identical activities of introduced PEPC. A preliminary analysis of transgenic potato and tobacco plants overexpressing up to six C₄-cycle genes will be presented. Bearing in mind that C₄-cycle enzymes are also present in C₃ plants, pitfalls encountered, when C₃ metabolism is perturbed by the overexpression of individual C₄ genes will be discussed.

P3.22—High level expression of C₄ enzymes in transgenic rice

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The recombinant DNA technology has made considerable progress in the molecular engineering of C₄ photosynthesis in the past several years, and it enabled us to overproduce enzymes involved in the C₄ photosynthesis pathway in desired locations in the leaves of C₃ plants. Phosphoenolpyruvate carboxylase (PEPC) and pyruvate, orthophosphate dikinase (PPDK) could be overproduced in the cytosol and the chloroplast, respectively, of rice leaves by introduction of the intact maize C₄-specific genes. NADP-malic enzyme (NADP-ME) could be overproduced in the chloroplast of rice leaves by introduction of cDNA for the maize C₄-specific NADP-ME fused to the rice *Cab* promoter. The maize PEPC and PPDK expressed in rice leaves underwent activity regulation through protein phosphorylation, and transgenic rice plants overproducing these enzymes grew normally. By contrast, overproduction of the maize NADP-ME led to serious stunting and leaf photobleaching of rice plants. These detrimental effects of the maize NADP-ME were ascribable to stimulated photoinhibition of photosynthesis due to an increase of the NADPH level in the chloroplast. Although overproduction of

either PEPC or PPDK did not improve the photosynthetic performance of rice plant, it had some physiological impacts on rice plants. Overproduction of PEPC enhanced the growth under nitrogen limiting conditions and also mitigated the inhibition of root elongation by aluminum in transgenic rice. Although preliminary, we observed that overproduction of PPDK increased the number of seeds per plant. It appears that these effects result from reinforced functions of PEPC and PPDK intrinsic to rice.

P3.23—Manipulation of Rubisco

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Genetic modification to increase the specificity of Rubisco for CO₂ relative to O₂ and to increase the catalytic rate of Rubisco in crop plants would have great agronomic importance. The availability of 3-dimensional structures of Rubisco at atomic resolution and the characterisation of site-directed mutants have greatly enhanced our understanding of the catalytic mechanism of Rubisco. However, knowledge-based alterations that can significantly improve the specificity and activity of Rubisco remain elusive. Considerable progress has been made in identifying natural variation in the catalytic properties of Rubisco from different species. Significant progress has also been made in developing the tools for introducing both novel and foreign Rubisco genes into plants. The additional complexities of assembling copies of the two distinct polypeptide subunits of Rubisco into a functional holoenzyme *in vivo* (requiring sufficient expression, post-translational modification, interaction with chaperonins and interaction with Rubisco activase) remain a major challenge.

P3.24—Limitations to CAM activity in a variegated cultivar of pineapple

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The pineapple, *Ananas comosus* (L.) Merr., is one of the most productive and economically important CAM plants, grown commercially over 60° of latitude. We are investigating physiological and metabolic attributes of this species which confer photosynthetic plasticity whilst maximising growth under the wide range of environmental conditions experienced in cultivation. In pineapple, the carbohydrate pool, which provides carbon skeletons for nocturnal organic acid accumulation, comprises of both starch and soluble sugars. The amount and type of carbohydrate present is known to vary under different environmental conditions. Currently we are interested in how carbohydrate availability influences CAM activity during long-term acclimation to contrasting light environments. The leaf organic acid content and levels of starch and soluble sugars were measured

in a variegated cultivar of *A. comosus* during acclimation of plants to high or low light conditions (800 μmol m⁻² s⁻¹ and 30 μmol m⁻² s⁻¹, respectively). Previous work has shown that the central, longitudinal band of green leaf tissue acts as a source of carbohydrate for the peripheral bands of yellow leaf tissue. During manipulations of both the environment and the source/sink relationship, changes in metabolites were related to the amount and activity of two key CAM enzymes, phosphoenolpyruvate carboxylase (PEPC) and phosphoenolpyruvate carboxykinase (PEPCK). The results of these investigations are discussed in terms of possible biochemical constraints on the functioning of CAM.

P3.25—Differential crassulacean acid metabolism (CAM) expression in four *Clusia* species: implications for PEP-carboxylase kinase genes

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The genus *Clusia* shows extraordinary diversity in terms of CAM expression. Species showing C₃-photosynthesis (*C. multiflora*), weak-CAM pattern with very small nocturnal CO₂-uptake (*C. aripoensis*), C₃/CAM intermediate pattern with C₃-type of day CO₂ up-take and CAM-type of night CO₂ up-take (*C. minor*) and constitutive CAM (*C. rosea*) exist. Comparing the mechanisms that underpin this metabolic diversity will help to identify the processes responsible for modulating CAM capacity and the resulting plasticity of the pathway. One of the most important features of CAM is the diel regulation of CO₂-uptake, which occurs mainly during the night. This regulation is due in part to changes in the properties of phosphoenolpyruvate carboxylase (PEPC) which are controlled by reversible phosphorylation. PEPC phosphorylation is achieved by a dedicated Ser/Thr protein kinase which leads to a more active enzyme at night having low allosteric inhibition by L-malate. PEPC-kinase is controlled by a circadian oscillator at the transcriptional level. Given the important role of this kinase in modulating PEPC activity during the diel cycle we have investigated the expression of PEPC-kinase genes in contrasting *Clusia* species under unstressed and drought conditions to modulate CAM expression. Two highly conserved *Ppc-k* genes are expressed in the four *Clusia* species and at least one of the two genes is induced by drought in all species. Differential splicing occurs for the two transcripts resulting into two different mRNA species for each gene. The 24 h expression patterns were compared for the two genes to the CO₂-uptake profiles in the different species.

P3.26—Physiological characterisation of a putative null CAM mutant of the model halophyte

Mesembryanthemum crystallinum

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In recent years, the model halophyte *Mesembryanthemum crystallinum* has come under increased scrutiny as a genetic model for elucidating mechanisms underlying salinity tolerance and the induction of crassulacean acid metabolism (CAM). *M. crystallinum* is particularly attractive for study due to its self-fertilisation with a relatively small genome, twice that of *Arabidopsis*, nine chromosomes (2N=18) and it shows developmentally regulated polyploidy. *M. crystallinum*, exhibits a rapid switch from C₃ photosynthesis into CAM in response to high salinity, easily identified by an accumulation of malic acid in the vacuole over the dark period. A recently isolated mutant of *M. crystallinum*, identified by its failure to conduct nocturnal acidification after exposure to salinity, has been obtained from mutant seed collections irradiated with fast neutrons and exposed to chemical mutagens. This putative null CAM mutant line provides an ideal opportunity to investigate signalling and mechanistic aspects of CAM induction. Here we present preliminary physiological data for both wild type and null-CAM mutant lines which were subjected to two treatments; well watered or exposure to 300 mM NaCl for 15 days. Data presented include time courses of gas exchange profiles, and changes in dawn /dusk titratable acidity, carbohydrates and polyols. This study gives us further insight into some of the factors influencing salinity tolerance and the induction of CAM.

P3.27—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. It catalyses a key step in photosynthetic CO₂ assimilation in some C₄ plants and Crassulacean Acid Metabolism plants (and algae) by participating in the CO₂ concentrating mechanism. It also plays a role in gluconeogenesis in C₃ plants. To elucidate the regulatory mechanism of PEPCK we have chosen to work with the C₄ plant *Panicum maximum*. 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 isolated a PEPCK gene fragment from *P. maximum* and are synthesising the cDNAs corresponding to the PEPCK gene using RACE PCR and primers corresponding to highly conserved regions downstream of the N-terminus. We have obtained approximately 1kb of the *P. maximum* PEPCK gene so far which is most similar to known C₄ PEPCK genes and less similar to C₃ PEPCK genes. We will use this approach to determine the full sequence of PEPCK and identify the potential phosphorylation site(s) for this enzyme. Also, we will use the cDNA for PEPCK to make a construct for over-expression in *E. coli* and by modifying the N-terminal sequence by site-directed mutagenesis we will determine the effect of phosphorylation on kinetic characteristics.

P3.28—Regulation of Rubisco in Crassulacean acid metabolism plants

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Rubisco in Crassulacean acid metabolism (CAM) plants should be co-regulated with the variable expression of gas-exchange patterns and the resulting large changes in the leaf internal CO₂ supply. Therefore, Rubisco regulation was investigated in the constitutive CAM species *Kalanchoe daigremontiana* in the context of gas exchange and fluorescence measurements. Well-watered plants were compared with drought-stressed plants, which did not show atmospheric CO₂ uptake at the end of the photoperiod. Differences in the diurnal pattern of gas-exchange and electron transport rate were mirrored by key factors of Rubisco regulation.

P3.29—Ecophysiology of *Tillandsia* epiphytes of a dry deciduous forest: seasonality and photosynthesis

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The present study evaluates the different seasonal performance in coexisting tank and atmospheric *Tillandsia* species of a dry deciduous forest that exhibits eight months without rain, during which dew deposition can be high and 95% of the trees are leafless. We quantified physiological, anatomical and architectural characters in the species *Tillandsia bartramii*, *T. drepanoclada*, *T. fasciculata*, *T. ionantha*, *T. pseudobaileyi* and *T. schiedeana*, to determine the different strategies these species are using to solve the same environmental problems. A positive correlation was found between annual carotenoid and malic acid in the different species, possibly due to the high requirements for photoprotection. Maximum malic acid concentration was observed at the beginning of the rainy season for the more exposed tank species,

whereas the atmospheric species presented their maximum peak in the dry season, after 90 days without rain. This could be a result of the higher resistance to drought and the possible capacity for water utilization from dew exhibited by the atmospheric species. Potentially intercepted light was calculated for the different plant orientations and leaf angles present in the species and it was found that regularly species with steeper leaf angles had less

trichome density in the adaxial surface than the species with lower leaf angles. *T. bartramii*, *T. drepanoclada* and *T. pseudobaileyi* presented preferential orientations that allowed them to avoid maximal radiation during drought. This study showed that these six species that share the same habitat have a different combination of strategies to maintain their photosynthetic activity in a changing environment.