Responses to Light in a Metabolic Model of Rice

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Responses to Light Intensity in a Genome–Scale Model of Rice Metabolism

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Analysis of a genome-scale metabolic of rice shows numerous coordinated changes between chloroplast and mitochondrial reactions in response to alteration in available light.

Abstract

We describe the construction and analysis of a genome-scale metabolic model representing a developing leaf cell of rice, *Oryza sativa*, primarily derived from the annotations in the RiceCyc database. We used Flux Balance Analysis to determine that the model represents a network capable of producing biomass precursors (amino-acids, nucleotides, lipid, starch, cellulose and lignin) in experimentally reported proportions, using CO2 as the sole carbon source. We then repeated the analysis over a range of photon flux values to examine responses in the solutions. The resulting flux distributions show that 1) redox shuttles between the chloroplast, cytosol and mitochondrion may play a significant role at low light levels; 2) Photorespiration can act to dissipate excess energy at high light-levels; 3) The role of mitochondrial metabolism is likely to vary considerably according to the balance between energy demand and availability. It is notable that these organelle interactions, consistent with many experimental observations, arise solely as a result of the need for mass and energy balancing without any explicit assumptions concerning kinetic or other regulatory mechanisms.

Introduction

Rice (varieties of the species *Oryza sativa*) makes up nearly 20% of the total caloric intake for the human population as a whole; the income of more than 100 million households in developing countries depends on rice cultivation. Although rice yield has increased, though gradually more slowly, during the last four decades, the world population continues to grow while the land and water resources for cultivation are declining, leading to a need for high yielding, stress tolerant, nutrient rich rice cultivars (Nguyen & Ferrero, 2006).

Researchers are trying to meet the challenges of improving production in different ways. Some of the efforts are: (i) identifying the stress tolerant rice varieties and stress responsive genes (Xiang et al., 2007); (ii) producing a "Green Super Rice" combining in a single plant many different favourable characteristics from the large number of available strains and cultivars, guided by molecular marker based selection (Zhang, 2007); (iii) introducing the genes of C4 plant to change the leaf anatomy of rice and hence improving the photosynthesis (Kajala et al., 2011), and (iv) producing vitamin-A enriched golden rice (Al-Babili & Beyer, 2005). In addition, current research on the genetic basis of signalling between nitrogen-fixing soil bacteria and legumes (Xie et al., 2012) has the potential to allow the engineering of nodule formation in cereal crops such as rice and wheat.

Here we present a genome scale model of rice metabolism and examine its responses to changing light availability. Since rice is also a model organism for other cereal crops such as wheat, the present effort should help researchers to understand the biochemistry of a photosynthetic crop plant as well as to compare it with other plants. In addition, the metabolic model of rice, which is the second metabolic model of a crop plant, can be used as a template for comparing the metabolism of different varieties of rice that are pathogen tolerant, drought tolerant, lower yield or higher yield, etc. and thus it may also help in identifying characteristics of individual varieties that may assist rice biotechnologists to breed the desired rice crop.
Modelling Metabolism

Modelling approaches

There are two broad classes of metabolic model: those that depend upon a knowledge of enzyme kinetics (kinetic models) and those that do not (structural models) (Schuster & Fell, 2007). Another distinction can be made between small metabolic models constructed manually to represent a small section of metabolism and large models that seek to represent the entire metabolic capabilities of an organism, built from data extracted from databases and termed genome–scale metabolic models (GSMs). The difficulty of obtaining kinetic equations and parameters for all of the enzymes and transporters involved in a genome–scale model, in spite of the development of robots for performing large numbers of enzyme measurements (Gibon et al., 2004), means that all such models investigated to date are structural.

A further advantage of structural modelling over kinetic modelling is that the structure of the network is completely defined by the set of reactions involved and their stoichiometric chemical equations. For example, the number of independent fluxes in a metabolic network, and the relationships between them at steady state, is can be determined from the null space of the stoichiometry matrix (Reder, 1988; Schuster & Schuster, 1989), and those metabolite concentrations constrained by mass conservation can be determined from the left null space of the stoichiometry matrix (Reder, 1988; Schuster & Höfer, 1991).

One early development was the use of linear programming to examine the constraints on metabolic function and product yields set by reaction stoichiometry and network structure (Fell & Small, 1986; Varma & Palsson, 1994), the methodology eventually becoming known by the term flux balance analysis (FBA) coined by the Palsson group. This type of computational analysis is a more efficient and thorough replacement for older conventional approaches, which consisted of manual accounting of the stoichiometry of metabolic conversions along defined pathways. Later, the development of elementary modes analysis (Schuster et al., 1999, 2000) showed that even small subsets of cellular metabolic networks could have many more paths and functions than are described as pathways in textbooks. Palsson and co–workers (e.g. Edwards & Palsson, 2000) pioneered the construction and analysis of metabolic networks consisting of the reactions catalysed by the enzymes contained in the annotations of whole genomes, beginning with Escherichia coli. Though the linear programming / FBA methodology, and certain other techniques such as null space analysis, scale easily to cope with the few thousand reactions of a GSM, elementary modes analysis undergoes a combinatorial explosion of enumerable pathways, and interpretation of the output becomes challenging, though not impossible (Carlson, 2009).

Though other methods have been proposed for finding feasible routes through metabolic networks, unless they explicitly take into account the conservation of mass throughout the network, represented by the stoichiometric constraints, (as do elementary modes analysis and FBA) then the routes will not be functional on a sustained basis (de Figueiredo et al., 2009). With the appropriate methods, it is possible to find the maximum yield of metabolic products from nutrients for a given metabolic network, and how this might be altered by knocking out certain enzyme reactions or introducing novel enzymes into the network (e.g. Trinh et al., 2008). Combined with some experimentally derived constraints, such as the maximum uptake rate of a nutrient, the rate of growth, and the composition of the organism’s biomass, FBA can predict an optimal set of internal fluxes within the network, and these predictions have been shown to be close to measurements made by metabolic flux analysis (e.g. Lee et al., 2008; Schuetz et al., 2007; Williams et al., 2010).

GSMs and their analysis

Currently there are approximately one hundred published GSMs of varying completeness and quality, plus several hundred existing as automatically–generated draft reconstructions (Henry et al., 2010). The major assumption in their analysis, as mentioned above, is that the production and consumption of all internal metabolites is stoichiometrically balanced, so their concentrations are constant and the system is at a steady state. Additional constraints are placed on the models, such as specifying maximum...
production rates of biomass components and/or nutrient uptake that are consistent with experimental observations. A common requirement in their analysis is the setting of an objective function that is optimised by the linear programming to produce a prediction of the fluxes in the network as nutrients are converted into excretory products and biomass. Most commonly, the objective function is to maximise the biomass yield from the nutrients used (Schuster et al., 2008), which has been shown to be appropriate for E. coli growing in a chemostat (Fong et al., 2003; Lewis et al., 2010), but which does not always reflect the use of fermentative catabolism unless additional constraints are set (Teusink et al., 2009). Another possible objective function is the minimisation of total flux in the system (Holzhütter, 2004; Poolman et al., 2009), which represents economy in the enzymic machinery.

A major application of GSMs is investigating and generating hypotheses about the operating characteristics of metabolic networks, represented by the differences between flux patterns in the network if control mechanisms operate to implement the different objectives mentioned above. GSMs also have the potential to be used as a framework for integrating and interpreting other types of high-throughput data, such as gene expression and proteomics measurements (Shlomi et al., 2007; Oberhardt et al., 2009), if the data can be incorporated into either the constraints or the objective function.

Eukaryotic GSMs

The majority of GSMs to date are of prokaryotes. The first eukaryotic GSM was that of Saccharomyces cerevisiae (Förster et al., 2003), which has gone through several iterations (Heavner et al., 2012) and has been followed by models of several other yeasts. The metabolic networks of human (Mo et al., 2007) and mouse (Selvarasu et al., 2010) exist as reconstructions, that is, as a compendium of reactions with gene associations en route to becoming a computable model. Photosynthetic organisms are represented at the microalgal level by Chlamydomonas reinhardtii (Boyle & Morgan, 2009; Chang et al., 2011) and Ostreococcus (Krumholz et al., 2012). For plants there are now several models of Arabidopsis thaliana (Poolman et al., 2009; de Oliveira Dal’Molin et al., 2010a; Mintz-Oron et al., 2012), and these are currently undergoing a process of collaborative reconciliation. The model of C4 metabolism for maize, sorghum and sugar cane (de Oliveira Dal’Molin et al., 2010b) was in fact derived from the Arabidopsis model rather than from the maize genome but a model based on the Zea mays genome was published shortly after (Saha et al., 2011). Hence to date there is only one GSM of a true crop plant (maize).

All the eukaryotic models vary in the extent to which cellular compartmentation is explicitly represented, and this remains a difficult problem owing to the incomplete and contradictory information about the localisation of the enzymes and the specificity of intracellular transporters. This is most marked in plants, which have specialised metabolic roles for cytosol, mitochondria, chloroplasts and peroxisomes (Seaver et al., 2012). Furthermore, whereas microbial metabolism can be analysed on the assumption that it is directed towards efficient growth (Schuster et al., 2008), other imperatives shape the metabolism of multicellular organisms, such as the need for mutual support of different tissues that implement different subsets of the genomically–encoded metabolic network by tissue–specific gene expression (Seaver et al., 2012).

A GSM for rice

In this paper we describe a GSM for rice, derived from the “RiceCyc” database (Youens-Clark et al., 2011), representing a network capable of the photo-autotrophic production of all major biomass precursors (carbohydrate, amino acids, nucleotide bases etc.) in experimentally observed proportion.

We analyse the model to identify feasible changes in reaction flux distribution in response to changes in photon flux, with the goal of identifying coordinated response across the whole system. It is clear that there must be some positive minimum value of photon flux below which precursor synthesis is not possible at the rate specified, and another, higher value, beyond which the additional energy cannot put to any use and must therefore be dissipated by the system. Our interest is to investigate how the metabolic network and its energy transduction mechanisms, particularly the mitochondrion, respond as
photon flux varies from minimal sustainable to hyper-abundance. Shastri & Morgan (2005) have previously examined dependence of photosynthetic metabolism on light energy, but in the prokaryote *Synechocystis*. The investigation of the *C. reinhardtii* GSM (Chang et al., 2011) was somewhat different as a more detailed model of the light reactions was used in order to model the dependence of growth rate on light quality.

A very similar approach (identifying response to changes in energy demand in heterotrophic plant cells) has been validated experimentally (Williams et al., 2010) using $^{13}$C MFA.

In our analysis we additionally consider the consequences of saturation of the Calvin cycle, here represented by an imposition of an upper limit on the sum of fluxes in the rubisco carboxylation and oxygenation reactions which also captures the effect of competition between CO$_2$ and O$_2$. This modelling assumption remains valid even if in reality other Calvin cycle reactions effectively limit the rate of assimilation.

The exact choice of the value for this upper limit is arbitrary (but was chosen such that its effects are only discernible at a photon flux above that where the metabolic state had stabilised) and allows us to explore responses at much higher light intensities where the chloroplast could become over-energised.

**Results**

**General model properties**

The initial reconstruction from RiceCyc generated a model with 1484 metabolites in 1736 reactions, of which 1029 could carry flux from nutrients to the specified biomass components, and at least 790 reactions had gene associations. (The gene-reaction associations are under-estimated, since we have not included counts for the manually added reactions such as the lumped light reactions and the compartmented mitochondrial and chloroplast metabolism). When optimal flux distributions were obtained over the range of light fluxes tested, 309 reactions were used in at least some part of the range.

**General responses to varying light levels**

We repeatedly solved the linear program described by equation 1, incrementing the the constraint representing the incident photon flux for each solution. In this way we accumulated a set of solutions allowing us to plot the fluxes associated with individual reactions as a function of photon flux.

The minimum photon flux for which a solution to equation 1 (see Methods) could be found was 0.321 light flux units, corresponding to a maximally efficient quantum demand of 13.4 photons per C fixed. The Assimilation Quotient (AQ, CO$_2$ fixed per O$_2$ released — the reciprocal of the Photosynthetic Quotient, PQ) at this point is 0.98, corresponding to 13.1 photons per O$_2$ released. As the light levels increase, the AQ falls in stages to a minimum of 0.87 then remains constant at this value.

The number of reactions being utilised at any particular photon flux across the range varied between 270 and 277. Of these 142 responded to changing light flux, all but 10 of which carried flux over the entire range of light flux values. The remaining 167 remained constant, reflecting the constant ratios of biomass components.

Above the threshold of minimal light flux, the objective value increases, to all intents and purposes, linearly (not shown). However, the responses of individual reactions are far more varied. Fig. 1 shows the distribution of correlations of reaction fluxes with photon flux. The strong responders are obviously highly coupled to the light reactions and involve transmission of reductant to the rest of the cell. The weak and moderate responders represent buffering between the strong responders and the constant biomass production. An examination of the reactions showing changes reveals that this is associated with...
shifting patterns of interaction between chloroplast and mitochondrial metabolism, as described below.

Specific responses to varying light

The changing interactions between chloroplast and mitochondrial metabolism are shown in Figs. 2 and 3. Across the range of photon fluxes scanned, five major metabolic patterns can be seen (A–E), with transitions between them occurring at the points where the set of reactions in the optimal solution changes, as indicated by the vertical boundaries between the regions. In regions A and B ATP is generated by the mitochondria (shown by the flux in Complex V, the ATP synthase), falling away in region C. The ATP synthesis is driven by carbon and reductant exported from the chloroplast, hence there are corresponding changes in the chloroplast transport reactions. A corollary is that some of the photosynthetic O2 is utilised by mitochondrial respiration, and conversely, the CO2 evolved by respiration is refixed in the chloroplast. The small fluctuations in reaction rates near the A–B border are associated with shifts in N–metabolism, as described later.

Mitochondrial response

The changing flux distributions shown in Figs. 2 and 3, correspond to various modes of operation of the mitochondrial metabolism presented in Fig. 4.

In region A, most flux is accounted for by generation of ATP through the operation of the electron transport chain with reductant supplied primarily from the operation of a mitochondrial malate-oxaloacetate shunt, with only a small contribution from the partial oxidation of pyruvate. Overlaid on this is a much smaller flux generating 2–oxoglutarate from fumarate and pyruvate, and the TCA cycle between 2–oxoglutarate fumarate, including Complex II (succinate dehydrogenase) is inactive. As region A is traversed, the flux patterns seen in region B gradually emerge.

Throughout region B, pyruvate is completely oxidised and most flux is accounted for by the conventional operation of the citric acid cycle and the electron transport chain, including complex II, leading to an increased synthesis of ATP, at a near–constant rate throughout, and the maximum reoxidation of photosynthetic assimilate to CO2. In addition to this, there is a small uptake of fumarate and malate, which now accounts for the export of 2–oxoglutarate at the same rate as in A. The malate-oxaloacetate shunt is inactive.

Region D (C is a transition between B and D) represents an unchanging state in which most of the cell’s energetic requirements are met externally to the mitochondria. The electron transport chain again operates, but without complex II and with much lower flux than previously: ATP production is reduced to less than 1% of that in regions A and B and oxygen consumption is minimal. Reductant is generated by the reactions of the TCA cycle from fumarase to isocitrate dehydrogenase oxidising fumarate, oxaloacetate and pyruvate to generate 2–oxoglutarate.

Region E represents minimum activity of the mitochondrion (also corresponding to the saturation of both the carboxylase and oxygenase reactions of Rubisco). Here oxaloacetate and pyruvate are still being incompletely oxidised to generate 2–oxoglutarate, with the resulting NADH being reoxidised via the operation of an incomplete electron transport chain comprising complexes I and V with the addition of the alternative oxidase reaction. It is only in this region that the alternative oxidase reaction is active, and it is the sole source of electron transport to the respiratory chain.
Chloroplast response

In region A, there is a glyceraldehyde 3–phosphate exchange with 3–phosphoglycerate across the chloroplast membrane, resulting in the transfer of reducing power from the chloroplast to the cytosol where oxaloacetate is reduced to malate that is taken up by the mitochondria. It is interesting to note that, although the chloroplast module contains a functioning malate–oxaloacetate transporter, this redox shuttle mechanism was not utilised in any of the solutions described here. Although the model represented by Eqn. 1 remains soluble if a flux is explicitly set in these reactions, all subsequent solutions are then suboptimal. As the light intensity increases towards region B, the import of 3–phosphoglycerate declines, as it is converted in the cytosol to the pyruvate that is taken up by the mitochondria. Also, the gross CO2 fixation rate increases as more is recycled from rising mitochondrial oxidation of pyruvate.

Throughout region B, the chloroplast metabolism, like that of the mitochondria, remains largely constant, until the transition to region C where mitochondrial ATP production starts to decline in the face of the increasing production of ATP by the light reactions, evidenced by an increase in oxygen evolution from the light reactions. The ATP is exported from the chloroplast to the cytosol by the restarting of the 3–phosphoglycerate/ glyceraldehyde 3–phosphate shuttle, generating ATP at the phosphoglycerate kinase reaction. The associated extra cytosolic reducing potential is accounted for by reactions associated with photorespiration, which becomes active at this point with an increasing export of phosphoglycollate throughout region C. The gross rate of CO2 fixation increases as that released by photorespiration more than replaces the declining mitochondrial production. Net evolution of O2 from the chloroplast increases in spite of its utilisation in the rubisco oxygenase reaction, but this is balanced by increased consumption in the rest of the cell as phosphoglycolate is recycled.

Across region D, photorespiration continues to increase, accompanied by increasing exchange in the 3–phosphoglycerate/glyceraldehyde 3–phosphate shuttle. The light reactions generate more O2, which is used in the photorespiration pathway. There is also an increased return of CO2 to the chloroplast from photorespiration with a further increase in the gross rate of assimilation.

In region E, rubisco has become saturated for both carboxylation and the oxygenase reaction. Continued increases in the light reactions result in a further increase in export of O2 from the chloroplast to the cytosol where it causes oxidation of ascorbate, and reductant is exported from the chloroplast to reduce the dehydroascorbate. Substrate cycling by the simultaneous polymerization and hydrolysis of starch is one of the ways the excess chloroplastic ATP is dissipated.

N uptake response

[Figure 5 about here.]

In our model, we consider that the rice leaf metabolism can take both NH3 and NO3 as nitrogen sources. Fig. 5 shows that there is a transition between NH3 and NO3 utilisation that takes place within region A of Fig. 2. At the very low intensity of light, the model favours NH3 over NO3 (but utilises both), then the utilisation of NH3 decreases and the NO3 increases, and finally, at photon flux values above 0.4, only NO3 is consumed. Since the use of NH3 can save energy in comparison to NO3, plant leaves may favour the utilisation of NH3 at low light intensities to optimise the energy available for biosynthesis. Indeed, the shift to NO3 utilisation is accompanied by a fall in AQ from 0.98 to 0.87, and at this point the quantum demand is 16.6 photons per fixed C. This certainly reflects the generation of the additional reductant needed for the assimilation of NO3.
Discussion

General model properties

Reaction Utilisation

An effect of minimising total flux in the network is to tend to also minimise the total number of reactions used, because if there are two alternative routes to convert metabolite \( a \) into metabolite \( C \):

\[
\begin{align*}
A & \rightarrow C \\
A & \rightarrow B \rightarrow C
\end{align*}
\]

the first option will always minimise total flux. This is not to say that the number of reactions found by this approach is the absolute minimum, but it does serve to place an upper limit upon that minimum.

The fact that only 309 of the 1029 available reactions were utilised in the solutions found here is therefore unsurprising and comparable with our previous study of heterotrophic metabolism in *A. thaliana* poolman *et al.* (2009), in which 232 out of 1406 available reactions were used. In addition, the solutions refer to only one physiological state of the cell, and a different set of reactions would be used during night–time metabolism. The number of reactions used here can be associated with a total of 568 unique genes, although this figure is certainly an underestimate, as mitochondrial and chloroplast reactions were not represented with BioCyc IDs.

Comparison with other models.

Our analysis is not readily comparable with that of previous plant GSMs (poolman *et al.*, 2009; de oliveira dal Molin *et al.*, 2010a; Mintz-Oron *et al.*, 2012; de oliveira Dal Molin *et al.*, 2010b; Saha *et al.*, 2011) as they have not considered a detailed biochemical analysis of the responses to light intensity. For example, Saha *et al.* (2011) considered two fixed photosynthetic states of maize, dependent solely on non–cyclic photophosphorylation, and concentrated on intercellular interactions of C4 metabolism and the properties of mutants in biosynthetic pathways. Conceptually our approach is closer to that of Shastri & Morgan (2005), but as their subject was the cyanobacterium Synechocystis, the lack of intracellular compartmentation and the different mode of life limit the meaningfulness of detailed comparison.

Boyle & Morgan (2009) reported results for three states of their *C. reinhardtii* model (autotrophic, heterotrophic and mixotrophic) and carried out a light intensity scan for mixotrophic growth with acetate, but this differs in many details from plant metabolism. Chang *et al.* (2011) studied the response of *C. reinhardtii* to light in great detail, but in the biotechnological context of predicting the growth rate expected given the spectral characteristics of the light source.

In effect, the diversity of the studies to date indicates the wide range of potential applications for genome–scale metabolic modelling and hints at the scope for further development.

Utility of LP constraint scanning.

The results presented here support our original contention that scanning constraints, as described in the Methods, does indeed reveal potential correlated responses in a network, and such observations can be used to generate further hypotheses concerning the in vivo behaviour of the system. This continues an approach adopted in the early studies of *E. coli* metabolism by FBA (Varma & Palsson, 1993; Varma *et al.*, 1993) before the model was genome–derived, where a series of different metabolic phases were identified by scanning the oxygen supply rate. Here we have restricted ourselves to one dimension by
fixing the biomass production rate, so that we essentially consider the ratio of light intensity to growth rate, which still gives us a challenging and rich behaviour to analyse. In the future, we need to consider different phases in the space defined by variation in light, growth rate, starch deposition and sucrose accumulation.

It is particularly interesting that simply scanning the light intensity results in the rubisco oxygenase reaction becoming active at super-optimal photon fluxes. This is, of course, a well known phenomenon, but there are no explicit or implicit features in the model or its analysis that make this a forgone conclusion. The observation thus lends further credence to this approach for the analysis of genome-scale models.

Comparison of specific responses to light with experimental observations

Photorespiration

The fact that photorespiration is spontaneously activated at supra–optimal photon flux suggests that the inhibition of this pathway might not be wholly beneficial to the plant. Plants are always likely to be exposed to supra–optimal light and have to be able to dissipate excess energy to prevent damage and over–reduction of the chloroplast. There are several mechanisms that contribute to this, and our results support the view that photorespiration is amongst these (Padmasree et al., 2002). It increases the net supply of CO2 to the chloroplast and absorbs the extra O2, as the changes in their fluxes (seen in regions C and D of Fig. 2) occur with no net impact on the CO2 and O2 fluxes for the whole cell, as is further discussed in the next subsection. The substantial export of glyceraldehyde 3–phosphate and the import of 3–phosphoglycerate in regions C, D and E show how reductant and ATP are moved out of the over–energised chloroplast. The importance of mitochondrial metabolism in the light for maximisation of photosynthetic performance has been noted by a number of authors (e.g. Padmasree et al., 2002) and is apparent in our model as described further below, but we have under–represented its contribution to photorespiration since this version of the model is not fully compartmented and does not assign glycine oxidation to the mitochondrion.

Quantum Demand and Photosynthetic Yield

The relationships between light absorbed, CO2 fixed and O2 evolved are at the centre of agronomic and ecological assessments of the roles of photosynthetic organisms, but they are difficult to measure experimentally and the theoretical limiting values are likewise hard to calculate from first principles. Both approaches share some common problems: photosynthesis is potentially simultaneously supporting export and storage of assimilate, growth and cell maintenance. At the same time, photorespiration and mitochondrial respiration can be taking place with their own stoichiometries for CO2 and O2. Furthermore, the instantaneous and long–term, and leaf and whole plant values are of interest. Whilst metabolic modelling does not resolve the issue of the relative mix of the different processes, it does allow rapid calculation for different well–controlled scenarios, such as different proportions of biomass and exported assimilate. Our calculations are for an average cell in an expanding leaf that is not yet a major exporter of assimilate, so have more in common with algal cultures than mature leaf or whole plant measurements.

Previous calculations of the minimum quantum demand for photosynthetic assimilation (e.g. Raven, 1982; Pirt, 1986) have used older stoichiometries for cyclic and non-cyclic photophosphorylation and have accounted for fixing CO2 and nitrate or ammonia to biomass on the basis of pathway–based calculations of the corresponding ATP and NADPH demands for making the basic constituents. From the lowest light level at which biomass can be produced in Fig. 2, where ammonia is being used as the nitrogen source and photorespiration is not active, we compute a quantum demand of 13.4 mol.mol⁻¹ (of absorbed photons) with our assumed maintenance requirement. The point at which nitrate becomes the nitrogen source for biomass in Fig. 5 corresponds to a higher quantum demand of 16.6 mol.mol⁻¹. Raven (1982)’s pathway–based calculations of algal quantum demand led to an estimate
of 14.1, after correction by Pirt (1986), excluding maintenance and polymerisation costs, whilst Pirt himself computed a lower value of 7.6 on NO3, or 6.4 on NH3. Pirt (1986) reviewed measurements on algae and concluded that the minimal quantum demand (corrected for maintenance) was likely less than 8.0. Hence our network–based calculations are essentially similar. The quantum demand reported for the GSM of the cyanobacterium *Synechocystis* was 13.9–14.7 using nitrate as the nitrogen source (Nogales et al., 2012). Experimental measurements on mature (i.e. non-growing) leaves of C3 plants reviewed by Skillman (2008) gave an average value of 19.2 mol.mol−1, presumably with maintenance included, under ambient atmospheric conditions. In measurements where photorespiration was suppressed, the median measurement fell to 10.4, but with a wide variation depending on the methodology.

The simplistic view of photosynthesis is that the assimilation quotient, AQ, is 1, i.e. one CO2 fixed per O2 released, and this figure is often assumed in calculations of photosynthetic performance. As pointed out by several authors over many decades (e.g. Raven, 1982), this is naive and over–simplified, since the elemental composition of the majority of biomass components requires that more O2 must be released than CO2 fixed to balance the stoichiometric equations for their formation. For example, Raven (1982) computed an AQ of 0.71 for algal growth with NO3 as N–source, and 0.89 with NH3. Our corresponding values for rice leaf biomass are 0.87 and 0.98 respectively, reflecting the substantial proportion of cellulose. It is often assumed that mitochondrial respiration and photorespiration will have significant effects on the value of AQ (e.g. Skillman, 2008), but though it may seem surprising, even at the highest light levels in our model, neither changes in mitochondrial respiration nor photorespiration have any effect on AQ. We are conducting a more detailed examination of the reasons for this.

A number of experimental studies by Bloom and colleagues (Searles & Bloom, 2003; Cousins & Bloom, 2004; Rachmilevitch et al., 2004) showed that the AQ was about 0.1 higher for growth on NH3 compared with NO3 in ambient atmospheric conditions for wheat, maize, tomato and Arabidopsis, in agreement with our calculation for rice. However, the lower AQ for NO3 was not seen under conditions where photorespiration was suppressed, such as elevated CO2 or reduced O2, and indeed, NO3 assimilation was reduced. This led to the suggestion (Rachmilevitch et al., 2004) that NO3 assimilation depended on photorespiration. This is not supported by our model, which exhibits unchanged nitrogen uptake characteristics from those shown in Fig. 5 when the rubisco oxygenase reaction is blocked. This implies that such experimental observations do not reflect a stoichiometric coupling but a kinetic or regulatory interaction.

Mitochondria and Chloroplasts

The involvement of mitochondrial reactions in the solutions across the photon flux range (regions A–E in Fig. 2) is support for the accumulated evidence that mitochondrial respiration has a role in optimising photosynthetic performance (e.g. Padmasree et al., 2002). Indeed, a number of the aspects of plant mitochondrial metabolism in the light described in their paper are evident in our results. For example:

1. Oxidation of pyruvate and malate during photosynthesis (Fig. 4); prevention of over–reduction of the chloroplast, and support of maximal photosynthesis. At lower light levels (regions A-C) the oxidation of pyruvate and malate is associated with the generation of ATP to support cytosolic metabolism. For an electron pair moved between water and NAD(P)H, mitochondria can generate more ATP and are therefore more effective at re–balancing reductant and ATP requirements than chloroplasts. The mitochondrial electron transport chain is active at all light levels, and the reductant being used is derived from the chloroplast light reactions. At high light levels, the model shows a shift to use of the alternative oxidase. (The flux in Complex IV in region E goes to zero, Fig. 4, so that disposal of reductant is not linked to generation of ATP, which is now provided in excess by the chloroplast.) This is consistent with the experimental evidence that the alternative oxidase is necessary to protect against the harmful effects of excess light (Bartoli et al., 2005).

2. Export of 2–oxoglutarate derived from pyruvate and C4 precursors to support nitrogen metabolism and amino acid synthesis. This activity continues at all light levels in our scan, though this invariance is because we have modelled a constant biomass production. There is some experimental evidence for export of citrate and its conversion to 2-oxoglutarate by the parallel
cytosolic reactions (Plaxton & Podestá, 2006), which we do not see in our solutions.

3. Operation of an incomplete TCA cycle. Succinate dehydrogenase (Complex II), and hence the complete TCA cycle, is only active at the lower light levels in regions A–C in Fig. 2, becoming active through region A and reaching its maximum level in region B before declining in C, whereas the other electron transport complexes and ATP synthesis continue at a low level in regions D and E. As a result, the responses to light intensity of the three neighbouring enzymes succinate dehydrogenase, fumarase and malate dehydrogenase are all different, as emphasised in Fig. 4. This ability of the TCA cycle reactions in plants to reconfigure to fulfil different needs has also been pointed out by other authors (e.g., Sweetlove et al., 2010), and, indeed, some of the flux patterns shown in that paper can be seen in Fig. 4. Also, the phenotypes of transgenic plants with reduced activities of succinate dehydrogenase and fumarase are different: tomato plants with fumarase reduced sufficiently to inhibit mitochondrial respiration have a reduced rate of photosynthesis and growth (Nunes-Nesi et al., 2007), whilst the opposite is the case for tomato and Arabidopsis with reduced succinate dehydrogenase (Araújo et al., 2011; Fuentes et al., 2011).

In both cases, the primary mechanism of the effect is reported to be through effects on stomatal conductance, possibly caused by metabolite signalling to the guard cells, rather than an effect on photosynthetic capacity. However, even if signalling is the dominant mechanism, the usefulness of fumarate and malate levels as signals could well be related to the reconfiguration of fluxes in this part of the TCA cycle at different light levels. Though succinate dehydrogenase is active in our optimal solutions obtained in light level regions A–C, it is not essential for biomass generation by photosynthesis since solutions, albeit sub-optimal, are obtained when its activity is deleted from the model.

4. Light inhibition of pyruvate dehydrogenase. Although this enzyme is present in solutions throughout the light range, its activity is at its maximum at the lower light levels of region B then falls away to the much lower level needed for synthesis of 2-oxoglutarate (see Fig. 2; it carries the same flux as the pyruvate transporter Pyr_tx).

It is, perhaps, surprising that the model can capture such a range of realistic behaviour of plant metabolism without having included any explicit regulatory mechanisms or enzyme kinetics. Pyruvate dehydrogenase offers an interesting illustration, as it is known to be inactivated by light (Padmasree et al., 2002). The assumption that metabolism will be optimal, combined with the stoichiometric constraints, leads to the conclusion that its activity should fall at high light levels, which implies the existence of a mechanism to achieve this without it having been specified in the model.

The results also show that the metabolic interactions between the plant organelles in the light are not fixed but shift according to the conditions. For this reason, we cannot expect to capture all the behaviour shown by a C3 plant in experiments, as we have so far only considered a leaf growing at a fixed rate with an adequate supply of CO2 and not yet exporting nutrients to the rest of the plant. Since a mature leaf can produce variable proportions of starch, sucrose and amino acids and operate at different levels of CO2 and light, we expect the model to reveal more potential for plasticity as we interrogate it in more detail.

There are aspects where we do not yet replicate some experimental observations reviewed in Padmasree et al. (2002). One comes from the representation of photorespiration. The model does not yet fully represent the compartmentation of the reactions involved, nor that the NADH generated by glycine decarboxylase is preferentially oxidised by mitochondrial pathways that do not generate ATP (Igamberdiev et al., 1997). Hence we do not show the total O2 consumption and CO2 production of the mitochondria at high light levels, nor the associated interchange of amino groups between mitochondria and chloroplasts.

Nitrogen assimilation

The model showed a transition between the use of NH3 and NO3 as light intensity increased from the lowest level able to support biomass formation. For our simulation, we assumed a fixed biomass generation rate and varied the light flux, hence the result more generally shows the trade-offs between nitrogen source, growth rate and photosynthetic rate. Because the photon demand is smaller for growth on...
NH3, wherever light intensity is limiting, faster growth will be possible using NH3 than NO3. At higher light intensities, the use of NO3 provides a sink for reductant generated by the excess photons. This illustrates the utility to the plant of having access to both nitrogen sources.

Conclusions

Overall, having added very few kinetic constraints to our stoichiometric model (saturation of rubisco - or the dark reactions of the Calvin cycle - and the limitation that cyclic photophosphorylation rate cannot exceed the non-cyclic rate) we find that realistic physiological behaviour emerges. This suggests that important aspects of plant photosynthetic metabolism are determined by stoichiometric imperatives, and that the specific kinetic and regulatory features are a way to satisfy these constraints and achieve a steady state. In turn, this implies that metabolism can only be fully understood at the whole network level, not as a mosaic of separate pathways.

Methods

Model construction and curation

Construction - general

The model was built using the same approach as for our previous model of A. thaliana (Poolman et al., 2009). An initial reaction set was generated from a publically available database. This set was curated and combined with a set of smaller sub-models (modules) defining various functionality not present in the set obtained from the database (described below).

The initial reaction set was extracted from the “RiceCyc” database downloaded from http://www.gramene.org/pathway/ricecyc.html (Youens-Clark et al., 2011) derived from International Rice Genome Sequencing Project’s annotation of the Oryza sativa L japonica cv. Nipponbare genome sequence. This database is available as part of the BioCyc collection (Karp et al., 2005).

The BioCyc format is particularly useful for metabolic reconstructions, as it clearly defines the hierarchical relationships between genes ↔ proteins ↔ enzymes ↔ reactions ↔ metabolites, and, where the database is under active curation by domain experts, these relationships get updated rather than being ‘frozen’ as ancillary data in the metabolic model.

The ScrumPy software package used here allows models to be defined in a modular manner such that logically separate groups of reactions are placed in separate files. This greatly facilitates data management and curation in situations where reactions defined in a database are combined with those from other sources.

In addition to the module defined by the reactions obtained from RiceCyc, two important extra modules were those defining the chloroplast and mitochondrial compartments. Other modules were included for convenience and are more completely described in the supplementary material S.1.

The Chloroplast module

The chloroplast module comprises the light reactions, Calvin cycle, the Rubisco oxygenase reaction (other reactions of photorespiration were treated as cytosolic as there is no explicit peroxisome compartment in this version of the model), starch metabolism, and reactions (that in the cytosol would be described as glycolytic) from PGA to Pyr, and malate dehydrogenase. Full details are given in the Supplementary Material S.1 and S.2.
The light reactions were represented as two lumped reactions, one for cyclic, and one for non-cyclic photophosphorylation. The stoichiometries were determined from the elementary modes analysis of a detailed model of the light reactions constructed on the basis of Allen’s (2003) stoichiometries of the underlying molecular processes. The equations used for cyclic and non-cyclic photophosphorylation were, respectively:

\[ 7 \nu + 3 \text{ADP} + 3 \text{P} \rightarrow 3 \text{ATP} \]

and

\[ 2\nu + 9 \text{ADP} + 7 \text{NADP} + 9 \text{P} \rightarrow 7 \text{O} + 7 \text{H}^+ + 7 \text{NADPH} + 9 \text{ATP} \]

In steady-state photosynthesis, non-cyclic and cyclic photophosphorylation are the major contributors in rice leaves (Makino et al., 2002). Though the water-water cycle (also known as the Mehler peroxidase reaction) can supply extra ATP needed in plant leaf metabolism, its maximum contribution is reported to be less than 5% of the linear electron flow in C3 leaves (Kramer & Evans, 2011; Ruuska et al., 2000). In addition, it is mainly functional during photosynthetic induction of rice leaves and not steady–state, so these reactions were not included.

Consideration was also given to the potential impact of a plastid terminal oxidase reaction (PTOX) Carol et al. (1999); Josse et al. (2000); Sun & Wen (2011) which acts to oxidise plastoquinol to plastoquinone, and has thus been proposed to act as a “safety-valve” under stress conditions.

When incorporated in the light reaction model, one new elementary mode was found, but the net stoichiometry of was identical to that of the cyclic photophosphorylation mode above. Therefore under the conditions investigated here, although PTOX may indeed carry flux, this will will not impact on the behaviour of the rest of the model.

The model used to generate these elementary modes is provided in the Supplementary Material S.4.

The Mitochondrial module

The mitochondrial module was that used and described by Poolman et al. (2009), and comprises the TCA cycle, ETC and oxidative phosphorylation; see also the supplementary material S.3 and figure 4(B).

Curation

In order for the results generated by a model to be of use, it is essential that a number of criteria are met; failure so to do would generate results implying violation of mass and/or energy conservation.

The first step in ensuring mass conservation is simply to determine the atomic balance of individual reactions. This check is readily achieved as BioCyc databases contain the empirical formulæ of most metabolites. Attention must then be turned to those for which no empirical formula is available. The commonest of these are polymers, and reactions involved with these must be treated with some caution if serious errors are to be avoided (Poolman et al., 2006). The problem arises when different reactions utilise different numbers of monomeric subunits: for example starch synthase catalyses the addition of a single glucose subunit to starch, but amylase removes two. The two reactions together would thus allow the generation of two glucose molecules from one. The issue can be easily resolved by defining the smallest monomeric subunit, and rescaling stoichiometric coefficients to reflect this. Thus the problem in the previous example may be resolved by considering glucose as the smallest monomeric sub-unit of starch and defining the amylase reaction to produce \( \frac{1}{2} \) molecule of maltose.

Energy and redox conservation may be readily checked using the linear program described in equation 1 below. All inputs and outputs are set to zero, and a demand for energy imposed in the form of flux in, e.g., a generic ATPase reaction.
incorrectly defined direction or reversibility. Happily, it is our experience that such solutions contain only a small number of reactions that may be conveniently checked against online databases or other sources.

Another potential problem related to reaction irreversibility is that of inconsistent subsets. In brief an enzyme (or reaction) subset is a set of (possibly not contiguous) reactions that carry steady state flux in a fixed ratio, a simple linear pathway being a trivial example (Pfeiffer et al., 1999). If two or more reactions in a subset have been defined as irreversible in opposing directions then no steady state flux is possible in the reactions comprising the subset. Subsets are identified from analysis of the null-space of the system, a subject beyond the scope of this paper, but see Fell et al. (2010) for further details. When inconsistent subsets are identified a judgement must be made as to the correct directionality of the irreversibility of the reactions within it.

A final check was to verify the stoichiometric consistency of the network with respect to C, N, P and S, as described by Gevorgyan et al. (2008).

Biomass composition

The model described here corresponds to a mesophyll cell in an expanding leaf, i.e. one that is producing cell components for a growing leaf, but not yet exporting material via the phloem to the rest of the plant. The relative proportions of the leaf components were taken from data by Juliano (1986), except for the nucleic acid content and composition, which were estimated from the results of Kwon & Soh (1985). The other biomass components were: amino acids for protein, plus β–alanine; nucleoside and deoxynucleoside monophosphates for DNA and RNA; polymerised glucose for starch and cellulose; coniferyl, coumaryl and sinapyl alcohols for lignin; linoleic acid for lipid and glucose and sucrose for soluble metabolites.

Biomass components were assigned an individual transporter, whose flux can be set independently, providing a more convenient mechanism to investigate variations in biomass composition than defining a single biomass reaction consuming all components, which has the disadvantage of making compositional variables into parameters as stoichiometric coefficients. The rates are represented as moles per arbitrary time unit and are given in the model files in the supplementary material (S.1).

An ATP hydrolysis reaction was included to represent the energy costs of polymerisation, turnover and cellular maintenance. In our previous application of genome–scale modelling to Arabidopsis root cells in culture (Poolman et al., 2009), we determined a value of 7.1 mmol.(g DW h)–1, which is in the range of experimental values reported for both bacteria and algae. This corresponded to approximately half the substrate input being used to meet this energy requirement, though this is probably higher than for a plant cell in its natural surroundings. Accordingly, we set the generic ATPase reaction at 0.1 light flux units which implies a photon demand of 0.1–0.2 light flux units depending on the routes used to generate the ATP.

Model Analysis

Apart from the determination of inconsistent subsets and stoichiometric consistency, a linear programming approach was taken for the major part of the analysis. This was defined as:

\[
\begin{align*}
\text{minimize} & \quad \mathbf{v} \\
\text{subject to} & \quad \mathbf{Nv} = \mathbf{0} \\
 & \quad \mathbf{v}_{i,j} = t \\
 & \quad \mathbf{v}_{\text{ATPase}} = \mathbf{ATPase} \\
 & \quad \mathbf{v}_p = \imath
\end{align*}
\]

(1)

Thus we minimise total flux, subject to a number of constraints: \( \mathbf{Nv} = \mathbf{0} \) defines steady-state, \( \mathbf{v}_{i,j} = t \) defines the constraints imposed by the requirement for individual biomass components (inputs of CO2 and inorganic nutrients along with the O2 output flux were left unconstrained), \( \mathbf{v}_{\text{ATPase}} = \mathbf{ATPase} \) defines the energy demand for polymerisation and cell maintenance, and \( \mathbf{v}_p = \mathbf{v} \) is the photon flux into the
A number of variants of this were used in the curation phase, for example, setting \( v_{i,j} \) and \( v \) to zero while setting ATPase to an arbitrary positive value, was used to check energetic consistency as described above.

**Light scanning**

As light intensity varies more rapidly (diurnal and transien) and over a much greater magnitude than the availability of CO2 or mineral nutrients this is the natural place in which to start our response analysis. Hence for this study we have analysed the responses of the solution of equation 1 to variation in \( v \). The range investigated was from zero (where, obviously no solution is found), up to the point beyond which all flux responses remain linear. The minimum flux below which no solution is possible was identified from a simple bisection search.

Two additional constraints were imposed for this aspect of the study: that cyclic photophosphorylation could not exceed non cyclic and an arbitrary limit set on the sum of the rubisco carboxylase and oxygenase reactions, to implement the limit on Calvin cycle flux as mentioned previously. To check the effect of the limitation on cyclic photophosphorylation, we repeated the light scans at different settings and this only affected the photon flux values at which the various transitions described in the Results occur, specifically the B to C transition. The key features of the flux distributions in the mitochondria and chloroplast remain.

**Software**

All computation was achieved using the software package ScrumPy - metabolic modelling in python Poolman (2006). This includes facilities for performing linear programming (using the Gnu Linear Programming Kit - [http://www.gnu.org/software/glpk/](http://www.gnu.org/software/glpk/)) and interrogating BioCyc flat-file databases.

The package, and further information can be obtained from [http://mudshark.brookes.ac.uk/ScrumPy](http://mudshark.brookes.ac.uk/ScrumPy) or by contacting MP.

[Table 1 about here.]

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Supplementary Material

Supplementary is distributed as a single tarred and gzipped (.tgz) archive containing two subdirectories, whose contents are described here

1. ‘Model’ directory containing the model in ScrumPy and sbml format with some explanatory text in a README file.
2. Diagram of chloroplast module in Model/Chloroplast.pdf. The ScrumPy module is in Model/Chloroplast.spy.
3. Mitochondrial module is to be found in Model/Mito.spy.
4. Details of calculation of net light reaction stoichiometries are found in the LightReac directory.

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Figure 1: **Reaction flux correlation with photon flux.** These are calculated as the absolute values (i.e. ignoring the distinction between correlated increases and decreases) of the pairwise (Peasons’s) correlation coefficients of each of the 142 variable fluxes with the photon flux across the range of light values. The reactions that show no flux changes are excluded.

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Figure 2: **Responses of solutions in the chloroplast and mitochondrion to varying photon fluxes.** Suffix _tx indicates transport of the named metabolite, positive values represent import to the compartment, negative values export. The abscissa is plotted as a logarithmic scale to enable the full set of responses to be easily seen; this causes the reaction responses to appear as curves, whereas they vary linearly with light intensity, showing abrupt changes in slope where the pattern of fluxes changes. The four major flux rearrangements are indicated by vertical lines, dividing the flux patterns into five major regions labelled A to E. The transition between regions A and B is shown at higher magnification in Fig. 3.

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Figure 3: **Chloroplast and mitochondrial transport responses at low light levels.** This shows a magnified view of the transition between the states A and B shown in figure 2. At lowest light levels there is a maximum import of 3-phosphoglycerate into the chloroplast, corresponding with the maximum
export of glyceraldehyde 3-phosphate, acting as a shuttle to export reductant and ATP. At the same point, the mitochondrial Malate-Oxaloacetate shuttle is maximally active as demonstrated by the import of malate, the activity of malate dehydrogenase and the export of oxaloacetate. Thus in this region, there is a net transfer of reductant from the chloroplast to the cytosol. The two curves for CO2 transport demonstrate the recycling from mitochondria to chloroplast.

Figure 4: **Flux distributions in the mitochondrial module at various photon fluxes.** Results are shown for specific flux values in the labelled regions as follows: A: 0.3, B: 0.8, D: 4.0, E: 8.0. State C (not shown) is intermediate between B and D. Flux figures are rounded to 1 sf for clarity. Reactions greyed out carry zero flux. In state B all reactions are active and this then describes the structure of the mitochondrial module.

Figure 5: **Variation in N source utilisation with varying photon flux.** Regions A and B are as shown in Fig 2. Note, however, the different photon flux range of this figure.

Table I: Abbreviations used in figures and elsewhere

| Metabolites          | Abbreviation     |
|----------------------|------------------|
| 2-OG                 | 2-oxo glutarate  |
| Cit                  | citrate          |
| CoA                  | coenzyme A       |
| Cyt_red/o            | cytochrome       |
| x                    | (reduced/oxidised) |
| Abbreviation | Full Name              |
|--------------|------------------------|
| Fum          | fumarate               |
| GAP          | glyceraldehyde 3-phosphate |
| IsoCit       | Iso-citrate            |
| Mal          | malate                 |
| OAA          | oxaloacetate           |
| PGA          | phosphoglycerate       |
| PGly         | phosphoglycolate       |
| Pyr          | pyruvate               |
| Q            | ubiquinol              |
| QH2          | ubiquinone             |
| Suc          | succinate              |
| SucCoA       | succinyl- coenzyme A   |
| **Other**    |                        |
| ETC          | Electron Transport Chain |
| MFA          | Metabolic Flux Analysis |
| MalDH        | malate dehydrogenase   |
I, II, III, IV, V : Complexes 1-5
r1 : Pyruvate dehydrogenase
r2 : Citrate synthase
r3 : Aconitase
r4 : Isocitrate dehydrogenase
r5 : 2-oxoglutarate dehydrogenase
r6 : Succinate thiokinase
r7 : Fumarase
r8 : Malate dehydrogenase
