Improving photosynthesis and yield potential in cereal crops by targeted genetic manipulation: prospects, progress and challenges

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Abstract

Since the ground breaking work of Norman Borlaug in the 1960's produced large increases in yields of our major cereal crops, we have seen a gradual decline in annual yield progress. The genetic potential of the yield components, harvest index and grain number, which were targeted in the "green revolution" and subsequently by cereal breeders have largely been optimised in our two largest global cereal crops, rice and wheat. Physiologists and breeders are turning to the biomass portion of the yield equation and in particular radiation use efficiency, as a means to push the yield potential barrier. Consequently, in the last decade a large effort has been initiated to identify targets to improve photosynthetic performance both using non-transgenic Phenomics approaches and transgenic technologies. Efficiency of light interception, harvesting and energy utilisation have been targeted but most efforts have so far focussed on improving photosynthetic capacity and efficiency in photosynthetic carbon metabolism in rice, wheat and model plants. Here the targets for improving light harvesting and carbon fixation are reviewed, the progress thus far evaluated and the likelihood of success of these activities in improving crop yields discussed in the context of modelling and scaling from the leaf to the canopy.

Introduction

Large increases in global rice and wheat production, which later came to be known as the Green Revolution, began in Mexico in the late 1950s, spread to Asia during the 1960s and 1970s, and then continued in China in the 1980s and 1990s. The benefits of this quantum leap in cereal production were to reduce prices of wheat and rice grain by more than 75% and over a 40-year period the proportion of the globe in famine declined from about 60% in 1960 to 17% in 2000 (Borlaug, 2007). While the fruits of the green revolution have sustained global demand for many years, annual increases in cereal grain yield from wheat and rice arising from genetic progress in breeding have now dropped below 1% (Brisson et al 2010; Sheehy and Mitchell 2011). Global population growth estimates indicate that by 2050 we will face the daunting prospect of feeding 9 billion people and even over the next 20 years, demand for cereal grain is likely to increase by 50% (FAO 2005, Borlaug 2007, FAO 2010; Rosegrant and Agcaoili 2010). To meet these burgeoning demands, quantum improvements in cereal grain yield are required and a coordinated effort to increase both genetic gain and efficiency of agronomic practices to reduce the “yield gap” is required (see Leegood et al, 2012).

A number of studies have been carried out in wheat to assess the yield components which had been targeted in the “Green Revolution” to produce the dramatic gains seen after introduction of the dwarfing genes to wheat and rice (Parry et al 2011). A large part of the progress in yield potential achieved during this time resulted from improvements in harvest index and grain number (ie carbon partitioning) rather than biomass (see Austin et al 1980; Fischer and Edmeades, 2010). However, there is evidence that this yield progress was also accompanied by higher photosynthetic rates on a leaf area basis and cooler canopies (Fischer et al 1998). Regardless of whether photosynthetic performance has tracked selection pressure on other traits, to date cereal breeding programs have not generally focussed on increasing yield via biomass improvement or radiation use efficiency (Evans et al 2012; Reynolds et al 2011). It is becoming apparent in both wheat and rice that yield progress through improvements in harvest index has been largely exhausted and that further improvements must come from the biomass portion of the yield equation (i.e. from improved radiation use efficiency and photosynthetic performance; see Sheehy and Mitchell 2011; Parry et al 2011).

Figure 1 summarises the physiological components of the yield and biomass equation, i.e. light interception and canopy level radiation use efficiency, and attempts to delineate in which part of the canopy these traits would have maximum impact. Most of these traits would be amenable to manipulation either by traditional/Phenomics screening based breeding approaches or candidate

gene approaches using recombinant DNA technology. Light interception of the crop is of pivotal importance in harvesting incoming radiation post canopy closure in cereal crops, as evidenced by a strong breeding focus on erectness in the current rice and winter wheat breeding ideotypes. With increasing erectness in plant type, distribution of photosynthetic machinery between leaves from the top to the base of the canopy must also change to accommodate improved penetration of light, but so far, modelling of canopy level photosynthesis in response to these parameters of architecture and leaf level biochemistry have not advanced rapidly (see Sheehy and Mitchell, this issue; DePury and Farquhar 1997; text below). The most intensively studied processes determining radiation use efficiency have been almost exclusively been measured at the leaf level. The biophysics of light harvesting, photosynthetic electron transport and the biochemical steps in C_3 photosynthesis are well understood and the steps controlling photosynthetic flux have been elucidated (reviewed in Zhu et al 2010 and Evans 2012). In many cases, candidate genes have been cloned and research has either demonstrated potential benefits in transgenic model plants or in some cases preliminary results have been presented in cereal crop species. The potential benefits of improved RUE on yield are evidenced by the positive effects of elevated CO_2 on crop plant yield potential (Ainsworth and Long 2005), comparisons of the yield of C_4 compared with C_3 crops (see Sheehy et al 2007; Parry et al 2011; Reynolds et al 2012) and have been modelled at the leaf level for many crops and for rice at the canopy level in this issue (Sheehy and Mitchell 2014).

This review details potential approaches to targeted genetic manipulation of canopy light interception and utilisation, and at the leaf level, follows potential routes to improvement along the path from light harvesting and chloroplast electron transport to carbon fixation and related biochemical processes.

Canopy architecture and leaf erectness

It has been shown that leaf erectness is an important trait in breeding for radiation use efficiency and there has been considerable success in breeding for more erect canopies in both rice and wheat (Yunusa et al 1993; Sinclair and Sheehy, 1999, Foulkes et al 2009). The available genetic variation in this trait raises the question of the requirement to engineer erectness. However, parameters for optimal canopy leaf angular distribution may shift as the canopy develops, with more prostrate leaves pre-canopy closure being useful in moisture conservation while in later growth stages, erectness of upper leaves may be crucial for light penetration. Canopy erectness has also been difficult to quantitatively measure making precision phenotyping challenging until the recent advent of canopy digitisation tools (Sirault et al 2013; Deery et al 2014). In rice, genes controlling leaf angle have recently been elucidated and manipulated (Zhao et al 2012) and QTL's identified in wheat

(Isidro et al 2012 and references therein). In rice, the *lc-1d* locus (Zhao et al 2012) encodes OsGH3-1, an indole-3-acetic acid (IAA) amido synthetase thought to act through IAA metabolism and brassinosteroid signalling. In the case of gain of function mutants in OsGH3-1 in rice, the leaf inclination phenotype appears to result from differential rates of growth of the adaxial and abaxial surfaces of the leaf at the laminar joint. While these data indicate that IAA homeostasis and possibly brassinosteroid signalling are important in genetic control of leaf angle, the genetic network in which OsGH3-1 sits appears to be large and complex, potentially involving GA (SPINDLY mutants; Shimada et al 2006), various MADS box transcription factors in the short vegetative phase group (Lee et al 2008) and a range of other genes expressed in the ligule (Lee et al 2007). While the genetic complexity of wheat has precluded cloning of candidate genes in the control of leaf angle thus far, QTL analysis (Isidro et al. 2012) indicates similar complexity in control of this trait to that in rice.

In conclusion, given **a.** the complexity of the genetic control of leaf angle **b.** the genetic variation already available in erectness in wheat and rice, and **c.** the lack of promoters available in cereals to drive tissue and developmental stage-specific gene expression, precise control of leaf angular distribution through transgenic approaches may not be tractable for some years to come. Progress in the shorter term may come from QTL mapping and possibly genotype by sequence approaches.

Optimisation of light harvesting and chloroplast electron transport

The inherent absolute quantum efficiency of C₃ photosynthesis, in the absence of restriction of energy utilisation by carbon metabolism or photorespiratory flux, has long been regarded as an immutable parameter (see Evans 1987, Singaas et al 2001). It is theoretically determined by the “simple” physics of how many photons are required to excite the photosystems for charge separation to produce sufficient electrons for reducing power and partition protons required to synthesise enough ATP for CO₂ fixation (for quantitative modelling, see von Caemmerer 2000). This view is supported by many surveys of photosynthetic quantum efficiency across a large number of C₃ species and genotypes under high CO₂ and unstressed conditions (Singaas et al 2001). Although the efficiency of light harvesting can be estimated from chlorophyll fluorescence parameters measured after a period in the dark (Fv/Fm; see Baker 2008) it is most often derived from photosynthetic rates of single leaves measured per unit light absorbed at limiting, i.e. very low, irradiance. Maximum photosynthetic quantum efficiency can be reduced under steady state conditions by a variety of processes, either via photoprotective energy dissipative mechanisms acting to protect photosystem 2 from over-excitation under high light or directly from photo-damage when these protective mechanisms are exhausted (Osmond and Forster 2008). Effective photochemical efficiency under steady state illumination can now be measured by a range of chlorophyll fluorescence techniques,

most notably Pulse Amplitude Modulated (PAM) fluorometry, using affordable commercially available tools (Baker 2008). PAM fluorometry enables the quantitative estimation of the proportion of absorbed light used to support photosynthetic flux (via photochemical quenching) and consequently electron transport rate, in addition to quantification of non-photochemical quenching of fluorescence, a measure of the energy dissipated in photo-protection and photo-damage (Baker 2008).

At a single leaf level, as irradiance is increased in a controlled experimental environment such as a CO₂ gas exchange cuvette or growth chamber, non-photochemical energy dissipation increases as the capacity for carbon fixation to utilise ATP and NADPH is reached for a given set of environmental conditions. The effective quantum efficiency of light harvesting is thus reduced to avoid photodamage. Repair of photodamage through resynthesis of the D1 protein, the most easily damaged component of PS2 light harvesting, is slow and energetically costly (Osmond and Forster 2008; Raven 2011). It has been postulated that this feedback regulation on light harvesting is in fact over-conservative, reacting inappropriately in annual crop plants and reducing the leaf level radiation use efficiency of cereals such as rice and wheat (Murchie and Niyogi 2011). In a field canopy, light flecks and rapid changes in ambient irradiance occur frequently due to wind and passing clouds, producing a light environment very different to that achieved in controlled environments. Once established, non-photochemical dissipation processes are slow to relax, taking from tens of minutes to hours depending on the nature of the mechanism (Murchie et al 2009) potentially preventing efficient utilisation of the lower steady state light levels pertinent to the lower parts of the canopy for much of the day. It is currently unclear how much genetic variation is present within a species in the capacity or rapidity of leaves to respond to and relax from over-energisation but interspecific variation in nature between high light and low light adapted species is well characterised.

Optimising light harvesting by genetic manipulation of photoprotection has been suggested for improvement of radiation use efficiency in cereal crops and theoretical evidence for the advantages of this approach has been reported (Zhu et al 2004, Murchie et al 2009, Murchie and Niyogi 2011, Reynolds et al 2012). A major component of non-photochemical energy dissipation in higher plants is mediated by the presence of pigments known as xanthophylls in the chloroplast thylakoid lumen (Demmig-Adams 1990). Zeaxanthin reduces the photochemical efficiency of PS2 by increasing energy dissipation as heat. The amount of Zeaxanthin present in the thylakoid is thought to be determined by the activity of the enzyme violaxanthin de-epoxidase, responsible for conversion of violaxanthin, an inactive form of the xanthophyll, to the active quenching form, zeaxanthin. This

dissipation mechanism has been well characterised in both eco-physiological and genetic studies (Demmig-Adams 1990, Niyogi et al 1998). The gene for violaxanthin de-epoxidase has been cloned from *Arabidopsis* (*npq1*) and mutants where this gene has been inactivated have been shown to be incapable of photoprotection (Niyogi et al 1998 and references therein). *Arabidopsis* mutants overproducing zeaxanthin due to a mutation in zeaxanthin epoxidase (*npq2*, allelic to *ABA1*, an ABA deficient mutant). Sequence for homologous genes in rice have also been identified and similar mutants are available in Tos17 insertion lines. At present, however, there is no clear strategy for altering the rate of response of zeaxanthin synthesis or breakdown in a fluctuating light environment. Impairment of photoprotection by genetic manipulation invariably results in photoinhibition and reduced plant performance (Niyogi et al 1998). Overproduction of Zeaxanthin, while altering the establishment and relaxation of non-photochemical dissipation, also affects ABA levels in leaf tissue producing pleiotropic phenotypes (Niyogi et al 1998). Precise quantitative engineering of these genes in crop plants will be necessary to achieve a positive benefit and differential expression in upper and lower leaves may also be necessary. In a related approach requiring precision engineering within the canopy, it has been suggested that reducing the investment in chlorophyll containing light harvesting complexes for leaves that are normally light saturated could improve light use efficiency per unit nitrogen investment in sunlit leaves (Zhu et al 2010). Similarly, reducing Rubisco investment in leaves which are light rather than Rubisco limited, could be beneficial to nitrogen use efficiency.

Recently another aspect of light harvesting has received considerable attention for potential improvement of photosynthesis: the spectral range over which chlorophyll can harvest light energy. The manipulation of leaf pigment composition by utilizing chlorophyll D for example would potentially increase the absorption spectrum of higher plant photosynthesis into the near infrared sufficiently to have positive benefits (Chen et al 2010, Chen and Blankenship 2011) however, the energy of light at these wavelengths is lower than in the normal visible spectrum. Generation of transgenic crops able to synthesise and utilise chlorophyll D would require cloning of the genes encoding the requisite biosynthetic steps (known in marine organisms; Chen et al 2010) and optimisation for expression in higher plants. In other, recent work researchers infiltrated higher plant plastids with nanotubes which improved the efficiency of light harvesting and electron transport significantly and similar effects could be demonstrated in leaf pieces (Giraldo et al 2014). At present, how this might be applied in a crop context is unclear but the results provide some optimism that enhancing energy harvested by the chloroplast may be possible.

Rubisco engineering

Rubisco is the enzyme which catalyses photosynthetic carbon fixation from the leaf intercellular spaces in all C_3 plants. Under saturating light the rate of photosynthesis is largely determined by the abundance, kinetic properties and activation state of Rubisco (von Caemmerer 2000) and under conditions of partial stomatal closure, due to abiotic stress or moderate irradiance, the bulk of control over photosynthetic flux also resides with Rubisco (von Caemmerer 2000). Rubisco is a remarkably slow catalyst and large amounts are needed to sustain high photosynthetic rates, for which reason this enzyme alone can account for more than 25% of leaf nitrogen (Parry et al., 2003). Increasing the abundance of Rubisco further, i.e. increasing photosynthetic capacity, would increase photosynthesis at high irradiance and high temperatures under ambient CO_2 concentrations (Yubuta et al., 2008). However, this approach would have limited application since it would require an increase in nitrogen fertiliser use and, unless Rubisco levels could be adjusted in leaves at different levels in the canopy, Rubisco would be in excess deeper in the canopy, decreasing crop nitrogen use efficiency.

Rubisco also catalyses a competing and wasteful reaction between the substrate RuBP and oxygen which leads to the loss of fixed carbon and nitrogen through photorespiration diverting captured energy away from carbon assimilation (reviewed in Parry et al 2011). C_3 crops such as wheat and rice waste approximately one third of Rubisco catalytic capacity on oxygenation and photorespiration in air at 25C, compromising radiation use efficiency (see von Caemmerer 2000). This wasteful oxygenase activity is also more pronounced when water supply is inadequate and stomata limit CO_2 diffusion into the leaf and when prevailing temperatures are high (which favours the oxygenation reaction over carboxylation). Early attempts to decrease or eliminate the oxygenase activity of Rubisco by site directed mutagenesis have so far been largely unsuccessful, however, it has been shown that mutated forms of Rubisco can be made and assembled in tobacco, including introduction of subunits from C_4 species (Whitney et al 2011b). It has also been shown in rice that introduction of a sorghum Rubisco small subunit by nuclear transformation produced a Rubisco with more C_4 like kinetic characteristics (Ishikawa et al 2011).

This field of Rubisco engineering is complicated by the fact that Rubisco in higher plants is comprised of two proteins; the large and small subunits (Whitney et al 2011). The large subunit is encoded by the chloroplast genome while the small subunit is nuclear encoded. While the nuclear encoded small subunit appears to control some aspects of Rubisco catalysis (see Whitney et al 2011a,b), the majority of catalytic properties appear to be related to sequence diversity in the chloroplast

encoded large subunit of C_3 plants. However, this is not the case in C_4 plants where specific amino acids have been identified to be responsible for variation in kinetic parameters (see Whitney et al 2011b). While transformation of the chloroplast genome is possible in some solanaceae, to date chloroplast transformation has not been reported for the major cereal crops. Replacement of Rubisco in a cereal crop with that of another species would be technically challenging, requiring context specific expression, assembly, post translational modification and regulation, in addition to transformation of the chloroplast genome. To achieve this, many technical hurdles need to be overcome (Parry et al., 2011). Nevertheless, the potential return for engineering Rubisco in a C_3 cereal crop would be considerable (see Zhu et al 2010; Parry et al 2011; Whitney et al 2011a). While surprisingly few higher plants have been surveyed for Rubisco kinetic properties, interspecies variation has been identified sufficient to suggest that it can be exploited as a means to improve crop photosynthesis. For example, replacing Rubisco in a cereal crop like wheat with that from the Mediterranean sea lavender, *Limonium gibertii*, would give significant increases in photosynthesis at concentrations of CO_2 up to current ambient levels (Parry et al 2011).

Engineering of Rubisco kinetics to improve photosynthesis remains a high return but long term strategy for cereal crops (see Parry et al 2011). While sequence comparison data and modelling provide clues concerning the parts of the Rubisco protein important in determining specificity and catalytic activity, no clear strategy for engineering improvement of the Rubisco protein has yet been reported and it may be that the two important properties of Rubisco targeted for improvement are inversely related, making Rubisco improvement challenging (see Whitney 2011). It is also likely that plastid transformation will also be required to deliver these improvements to cereals when demonstrated in model species.

CO₂ concentrating mechanisms: bicarbonate transporters

Over the past decade there has been intense interest in mimicking evolution by introducing a mechanism to concentrate CO_2 at the site of Rubisco. Increasing CO_2 around Rubisco can have a dual effect in reducing wasteful photorespiration and increasing the rate of catalysis of Rubisco as it operates closer to substrate saturation. CO_2 concentrating mechanisms, while not present in C_3 crops like rice and wheat, have evolved multiple times in both single cell organisms and higher plants, providing a genetic and biochemical model of how to install such mechanisms in C_3 cereals (see Parry et al 2011; Price et al 2011, 2013; von Caemmerer et al 2012). In the case of single celled photosynthetic organisms, a variety of membrane localised inorganic carbon transporters are believed to carry out this process (called inorganic carbon concentrating mechanisms or CCMs; Price

et al 2013) while in higher plants, a biochemical CO₂ concentrating mechanism such as C₄ photosynthesis has evolved in independent lineages more than 60 times in a variety of monocots and dicots (Sage et al 2012) and this is discussed further in the following section.

A variety of single cell microalgae and cyanobacteria living in low CO₂ environments contain membrane based inorganic carbon pumps to concentrate CO₂ around Rubisco providing a range of candidate genes for introduction of this process into higher plant chloroplasts (Price et al 2013). This approach might be less technically challenging than engineering a C₄ photosynthetic pathway due to the smaller number of genes required and also, the energy costs of a CCM may be inherently lower than that of the C₄ pathway (Price et al 2013). Single-subunit HCO₃⁻ transporters such as BicA and SbtA which have been well characterised in cyanobacteria are obvious choices, although multi-subunit transporters such as the BCT1 HCO₃⁻ transporter and NDH1-based CO₂ uptake systems could also be used (see Fig 2; Price *et al.*, 2008; 2014). Likewise, HCO₃⁻ transporters from micro-algae such as *Chlamydomonas* could also be considered as candidates for installation (Spalding, 2008).

Early work attempting to express a *Synechocystis* CCM in plants focused on the IctB gene, a membrane spanning protein and putative bicarbonate porter (Lieman-Hurwitz et al 2003). These authors transformed both Arabidopsis and tobacco with this gene by nuclear transformation and saw small but reproducible changes in photosynthetic gas exchange characteristics and growth. However, following discovery of the BicA proteins, the IctB protein has shown *not* to be a bicarbonate porter, making the physiological effects of its expression in higher plants, such as improved photosynthesis and growth, intriguing and difficult to explain (reviewed in Price et al 2013).

Recently, the first example of expression of the BicA class of bicarbonate porters in higher plants was reported where the gene was incorporated into the plastid genome of tobacco and the protein complex shown to be inserted into the chloroplast membrane at physiologically relevant levels *in vivo* (Pengelly et al 2014). Unfortunately, the tobacco transformants showed no physiological phenotype, including lack of a gas exchange and isotopic phenotype predicted from modelling, suggesting that the transporter was not active in these plants. The reasons for this lack of activity may be the lack of a sufficient Na⁺ gradient across the chloroplast envelope to drive the transporter (these proteins are Na⁺/HCO₃⁻ antiporters; Fig 2) or the requirement for post-translational activation, an explanation more favoured by the authors of the work (Pengelly et al 2014). It may be that a number of additional genes will be required to introduce an efficient and fully functional cyanobacterial CCM into crop plants but the modelled energetic benefits of such a system

(60% increase in maximum rates of photosynthesis and a 36% to 60% increase in yield) provides considerable promise (Price *et al.*, 2013; McGrath and Long 2014).

We are currently unable to accurately predict the likely benefits of introducing a CCM into higher plant chloroplasts due to a lack of knowledge of the diffusion properties of the chloroplast envelope, plasma membrane and cell wall to CO₂ (Evans *et al.*, 2009). If CO₂ could freely pass across the compartment where it is being concentrated and then diffuse back into the atmosphere, the energetic costs of the CCM could be too high to provide a benefit translatable to yield. Leaf morphology in C₃ plants appears to minimise the diffusion path for CO₂ from the intercellular spaces to Rubisco, a characteristic which may be counterproductive for plants concentrating CO₂ in the chloroplast compartment (see Evans *et al.*, 2009). Repackaging Rubisco in some type of carboxysome, as found in cyanobacteria, or pyrenoid structure, as found in certain algae, might be beneficial in achieving efficient operation of a CCM (Price *et al.* 2014). The carboxysome has been well characterised in cyanobacteria (reviewed in Rae *et al* 2013) and is a proteinaceous body which contains most of the Rubisco and CA (carbonic anhydrase; which interconverts bicarbonate and CO₂) present in the cyanobacterial cell. Sugar phosphates and bicarbonate can pass freely across the carboxysome but CO₂ diffuses out very poorly (Rae *et al* 2013; Price *et al* 2013). Thus, a significant elevation of CO₂ within the carboxysome is possible. There are more than 7 proteins required to assemble a carboxysome in cyanobacterial cell making this a challenging engineering problem to introduce such a structure to plants. Recently, 5 of the genes encoding the carboxysome shell proteins were transiently expressed using an agrobacterium mediated system in *Nicotiana*, producing carboxysome-like structures in the transformed leaf tissue (Lin *et al* 2014). While physiological analysis of function was not feasible in this system, it provides hope that introduction of a full carboxysome-based CCM into higher plants such as that shown in Fig 2 may be possible.

While plastid transformation was recently used to introduce BicA into higher plants, all the components of the cyanobacterial CCM, including the carboxysome shell are theoretically able to be transferred using nuclear transformation, although some codon usage optimisation may be required. Thus, the timeframe for introducing this mechanism to crops may be shorter than Rubisco engineering and the benefits would be of a similar magnitude by reducing photorespiratory losses and increasing Rubisco catalytic rates in air (see Parry *et al* 2011; Price *et al* 2013; McGrath and Long 2014). Installing bicarbonate transporters in higher plants is a major focus of a number of recently formed consortia for photosynthetic improvement including the Bill and Melinda Gates Foundation funded *RIPE* consortium. It is likely, given the intense activity in this area of research, that this strategy will soon be tested in cereal crop species.

CO₂ concentrating mechanisms: C₄ and C₄-like biochemical pumps

As pointed out above, mechanisms for elevation of CO₂ at the active site of Rubisco have also evolved in higher plants as a solution to the poor performance of this catalyst in air, and most of which are variants of the C₄ pathway, discovered more than 45 years ago (Hatch and Slack 1966). This pathway has evolved more than 60 times using a mixture of 3 major biochemical mechanisms, most commonly involving both biochemical and anatomical specialisation (Furbank 2011; Sage et al 2012). C₄ photosynthesis employs a “biochemical” CO₂ pump rather than a membrane based biophysical pump, using PEP carboxylase to fix CO₂ (as bicarbonate) in the mesophyll cells of the leaf (Fig 3). The resulting 4-carbon dicarboxylic acid or a derivative thereof (hence the label C₄) diffuses to specialised bundle sheath cells surrounding the vascular bundles (known as Kranz anatomy) where CO₂ is released enzymically to be fixed by Rubisco and a 3 carbon compound returned to the mesophyll to be processed to regenerate PEP for PEP carboxylase to act once more on atmospherically derived bicarbonate. The net result of this process is that CO₂ in the bundle sheath compartment of C₄ leaves can reach levels 10 fold atmospheric, eliminating photorespiratory energy losses and enabling Rubisco to operate at its catalytic optimum for CO₂ fixation (Sage and Zhu 2011 and references therein). Hence, the theoretical efficiency for conversion of total solar energy to plant biomass in a C₄ is 60% higher than in a C₃ cereal like rice or wheat, (see Zhu *et al.* 2008, Furbank et al 2009, Parry et al 2011; Sheehy and Mitchell, this issue). These effects on RUE when translated into biomass and yield potential are evident when we compare the biomass attainable in C₃ species with that of C₄'s for equivalent integrated thermal time (Byrt et al 2011); C₄ crop biomass can exceed the best C₃ values by up to 3 fold and if translated into yield, this improvement could also be compounded in the field by an impact on water and N-use efficiency, making the installation of C₄ photosynthesis or a modification thereof, an attractive proposition (see refs in Sheehy, Mitchell & Hardy 2007; Parry et al 2011, Sage and Zhu 2011). C₄ crop species include maize, sorghum and sugarcane (and some of the world's worst weeds) but unfortunately the number of commercially cultivated C₄ species is small. Perhaps part of the reason for this is the relatively recent evolution of C₄ photosynthesis and its absence in the clades of the major crops such as the Triticeae and Oryza tribes (Sage 2004).

Engineering biochemical C₄ pathways into C₃ crops both with and without Kranz anatomical specialisation is being attempted at present, focussing mostly on rice (von Caemmerer et al 2012; Kajala et al 2011; Miyao et al 2011). A full Kranz anatomy based C₄ system requires a considerable amount of gene discovery, genetic engineering and analysis of transgenic plants. The number of

times the C_4 pathway has evolved, however, has led to some optimism as to the likely success of this approach and there are a number of options for installation of C_4 pathway variants (Furbank 2011) and “intermediate” C_4 pathways in C_3 plants.

In 2008, the Bill and Melinda Gates Foundation funded a major initiative to build a C_4 pathway in rice based on a multiphase gene discovery and transgenic rice construction project to 1. Identify genes responsible for anatomical specialisation 2. Build a toolkit of gene constructs necessary to appropriately express enzymes and transporters in the mesophyll and bundle sheath cells of rice and 3. Install the enzymes and metabolite transporters in rice, sourcing many of the genes from maize then 4. Bring anatomical specialisation together with transgenic rice lines harbouring all genes for the biochemical pathway either by crossing or re-transformation (see Kajala et al 2011; von Caemmerer et al 2012). Following this prototype building phase (9 years in total) it is envisaged that fine tuning efficiencies and transferring the C_4 trait to high yielding adapted germplasm could take another 6-10 years.

The C_4 Rice Initiative has focussed on one of the three biochemical variants of the C_4 pathway known as NADP-ME type, common in many C_4 crop species (illustrated in Fig 3) and will attempt to recreate “Kranz” anatomy in rice, characterised by large bundle sheath cells encircling the vascular bundles, containing abundant chloroplasts where Rubisco will be exclusively localised (see Figs 3, 4; von Caemmerer et al 2012; Kajala et al 2011, Sage and Zhu 2011, von Caemmerer & Furbank 2003). At the beginning of the C_4 rice project, the NADPME-type C_4 mechanism was well understood and many of the genes encoding the necessary enzymes and transporters had been cloned. While the cis and trans acting factors controlling cell specific expression of these genes in mesophyll or bundle sheath have largely proven elusive (with one notable exception, MEM1, Akyildiz et al 2007), promoters from the PEPC gene of maize and a promoter from a PEPcarboxykinase gene from the C_4 grass *Zoysia japonica* have been shown to drive preferential expression in the mesophyll and bundle sheath cells of rice, respectively (Matsuoka et al 1994; Nomura et al 2005). These promoters have so far been routinely used in building the C_4 rice prototype. Recently, researchers in the consortium have assembled rice lines containing all 5 genes encoding C_4 pathway enzymes and expressing the proteins necessary to carry out a prototype pathway and these plants are currently being grown to homozygosity and are undergoing functional analysis. An example of the mesophyll cell preferential expression of the PEPC gene in transgenic rice leaves is shown in Fig 4.

Progress in determining the key genes necessary to introduce C_4 anatomy into a rice leaf has been slow. One of the most obvious differences in leaf anatomy between rice and a typical C_4 leaf is that

C₄ leaves seldom have more than 2 cells between bundle sheath cells and vascular bundles while C₃ monocots can have up to 9 (fig 4 and von Caemmerer et al 2012). Closer vein spacing is thought to be an important early step in the evolution of anatomical specialisation in Kranz C₄ photosynthesis (Sage et al 2012). In addition, bundle sheath cells in C₄ leaves are larger and have more chloroplasts than in rice and conversely, chloroplast numbers in C₄ mesophyll cells are generally lower than in rice mesophyll (see Fig 4). Due to the ease of visual screening, large populations of rice which have activation tags distributed throughout the genome or have genes from a large part of the genome individually inactivated have been screened for closer vein spacing. Very few candidate genes have been found from this approach, and those found tend to show a high degree of plasticity with environmental conditions. A mutational loss of function approach in Sorghum and the model C₄ grass *Setaria viridis* has been more productive and candidate genes are now under evaluation. In parallel, a large systems biology and bioinformatics effort has been made to identify gene candidates important for C₄ traits, including anatomical specialisation (Fouracre et al 2014), by 1. Comparison of gene expression patterns in leaves of closely related C₃ and C₄ plants (www.onekp.com); 2. Comparison of genome sequence information from closely related C₃ and C₄ species 3. Comparison of gene expression along a developmental gradient of maize leaves and rice leaves (Li et al 2010) and 4. Comparison of gene expression patterns in maize leaf primordia (leading to Kranz anatomy) and husk primordia (leading to non Kranz anatomy) (Wang et al 2013). From this large data set, a subset of approximately 150 gene candidates have been identified by filtering of these data sets and clustering analysis with expression patterns of genes known to be important in the C₄ pathway and differentially expressed in the two cell types. These candidates are being tested for function by gene inactivation in C₄ species and by overexpression in fast cycle (Kitaake) rice.

While validated C₄ anatomical regulatory genes have yet to emerge from these approaches, some recent progress has arisen from analysis of the “scarecrow” (Scr) / “short root” (Shr) mutants in maize (Slewinski et al 2012) and transcriptional analysis of Kranz and non-Kranz primordial tissue in maize (Wang et al 2013). The Scr/Shr pathway regulates radial patterning in the stem and roots of C₃ plants and it has been proposed that it was recruited into the leaf during evolution of Kranz anatomy in C₄ plants (Slewinski et al., 2012). The correlation between expression of the genes in this pathway in leaf primordial and Kranz anatomy together with the mutational analysis provide strong evidence for the involvement of the “short root” signal transduction pathway in C₄ development, however, gain of C₄ anatomical function in C₃ plants by manipulation of this pathway has not so far been demonstrated.

Although Kranz anatomy appears to have been the more common evolutionary route for high performing C_4 plants, in some cases this spatial separation of the biochemical CO_2 “pump” from the site of Rubisco can be achieved by spatial separation of the two types of chloroplasts within a single cell (reviewed in Edwards and Voznesenskaya 2011). Presumably some barrier to CO_2 diffusion exists within these cells to allow CO_2 to accumulate but little information is available as to the nature or effectiveness of this barrier. Similarly, in Kranz type C_4 plants, it is not known whether a barrier to CO_2 diffusion out of the bundle sheath cells at the level of a specialised cell wall or suberised lamella is necessary for the efficient operation of the C_4 pathway and modelling suggests that organelle localisation within the bundle sheath cells and diffusion path length may be equally important (see von Caemmerer and Furbank 2003). Information on the permeability of the bundle sheath cell wall in wheat and rice to CO_2 is currently unavailable. Similarly, chloroplast number and volume in the bundle sheath is considerably higher in C_4 than in C_3 cereals, presenting a potential obstacle for C_4 rice and wheat (See Fig 3; Sage and Zhu 2011).

RuBP regeneration and Photorespiratory “bypass”

Under conditions of moderate light, ambient CO_2 and when stomatal conductance is high, the control of photosynthetic flux is shared between the catalytic capacity of Rubisco and the capacity for the Calvin cycle to regenerate the substrate for Rubisco, RuBP (von Caemmerer 2000). The key rate limiting enzymes in the “regenerative” phase of the Calvin cycle have been identified and their levels manipulated (see Evans 2012). Over expression of sedoheptulose-1,7- biphosphatase (Lefebvre et al., 2005; Tamoi et al., 2006) or fructose 1,6-bisphosphate aldolase, (Haake et al., 1999) increase both photosynthetic rate and biomass accumulation in model plants under controlled environment conditions. Experiments are underway in wheat to validate these results in a cereal crop with gene constructs targeting both enzymes but only preliminary results are currently available (Raines and Parry, pers. com.). Numerical simulation using an evolutionary algorithm to optimize the distribution of resources between enzymes of carbon metabolism where leaf nitrogen levels are kept constant supports the experimental results that the levels of these enzymes are not optimised and further suggests that decreasing levels of some photorespiratory enzymes would also increase photosynthesis (Zhu et al., 2007). Further decreases in the photorespiratory pathway may be made if combined with a photorespiratory bypass (Kebeisch et al 2007; Peterhansel and Maurino 2011; Peterhansel et al 2012; Carvalho et al 2012). This bypass involves introducing bacterial enzymes to recycle photorespiratory products without releasing ammonia and in two of the 3 options which have been tested, CO_2 is released within the chloroplast compartment rather than the peroxisome (reviewed in Peterhansel et al 2012). This approach appeared to have a positive benefit

in *Arabidopsis* on both photosynthetic efficiency and biomass (Kebeisch et al 2007) but these observations have not been replicated to date in any crop species.

Developing an integrated canopy photosynthesis approach to scale across space and time

Sinclair et al (2004) argued that improvement to crop growth from changes to photosynthesis at the biochemical level was likely to be small. However, proving or disproving this hypothesis conclusively represents a significant challenge without an advanced modelling framework for integrating across spatial boundaries and temporal scales to assess the value of specific changes. Assessment of the impact of specific engineering strategies has largely been restricted to leaf level analyses and few attempts have been made to bring biochemical models together with plant and canopy growth and yield models. Canopy photosynthesis is an emergent property of not only the biochemical and structural characteristics of the leaves, but also of the physical arrangement of these leaves at the shoot-architectural scale and of their interactions (Chen et al, 1994). Building detailed dynamic mechanistic models (*cf.* Šafránek et al, 2011) incorporating biochemical, leaf and canopy level photosynthesis models with relevant feedback effects and structural information are of upmost importance for revealing the functional implications of the engineered modifications or manipulations discussed previously. This is particularly important when assessing the potential value of stacking multiple transgenes in a canopy environment. For example, Zhu and Long (2009) examined the current efforts to increasing photosynthetic rate by increasing the specificity of Rubisco for CO₂ relative to O₂ (τ). These authors modelled the effect of an increase in τ at the canopy level using a steady-state biochemical model of leaf photosynthesis (Farquhar et al, 1980; von Caemmerer, 2000) coupled to a canopy biophysical microclimate model (Norman, 1980; Zhu et al 2004). They concluded that an increase in τ , if achieved at the expense of the catalytic constant k_c , would result in decreased daily canopy carbon gain at the current atmospheric CO₂ level. Although this conclusion appears counter-intuitive, it can be explained by taking into account the heterogeneity of the light environment within canopies to assess the effect of a modification at the biochemical level. If one considers that crop canopies have both sunlit and shaded leaves, i.e. leaves in both light-limited and light-saturated conditions, it is easier to understand that an optimal τ needs to reflect the balance between the two photosynthetic limitations (light and Rubisco limitations) which occur concurrently in different leaves within a canopy (see fig 1).

Computational tools are now essential for predicting and analysing links between CO₂ and light capture for photosynthesis, crop growth and yield. These tools are used to provide a virtual environment for linking models describing the primary processes in thylakoid membranes such as light harvesting in photosystem I and photosystem II (Riznichenko and Rubin, 2007; van Grondelle et

al, 2009; Scholes et al, 2011), to models expressing CO₂ diffusion pathways in leaves by taking into account the specific anatomical arrangement and position of chloroplasts in leaves (Ho et al, 2012), to models capturing the key limiting biochemical processes in C₃ and C₄ photosynthetic pathways at the leaf level (Farquhar et al 1980; von Caemmerer, 2000; von Caemmerer and Furbank, 2003), to model scaling up from the leaf to the canopy (de Pury and Farquhar, 1997; Kobayashi et al, 2012). However, these models describing different spatial or temporal scales are yet to be aggregated. Two of the key challenges to developing a comprehensive model are 1. To transfer information from one scale to another (since photosynthesis does not scale linearly from the plastid to the canopy); and, 2. To model the phylloclimate around leaves (Chelle, 2005) since leaf photosynthesis also responds non-linearly to temperature, vapour pressure gradients and light. Efforts aimed at developing all-inclusive models capturing the salient features of canopy photosynthesis operating across the scales of biological organisation are underway (Nedbal et al, 2009; Zhu et al, 2010; Duursma and Medlyn, 2012) although such approaches have their opponents (Raupach MR and Finnigan, 1988). While such models are inherently complex due to the interplay of so many variables and perhaps of limited utility in an agronomy context, they are nonetheless pivotal for estimating the trade-offs associated with the modification of individual enzymes, genes and traits acting at lower levels. Lack of interoperability between models at different scales (chloroplast to leaf to whole plant to canopy) represents a major challenge to this field of research. A potential solution to this issue is the concept of 'Comprehensive Modelling Space' (Nedbal et al, 2009). The CMS is a framework where the partial photosynthesis models described above can be shared and integrated (Le Novere et al, 2005).

Whenever there is a need to quantify total biomass gain from photosynthesis, an accurate description of plant architecture and leaf position is required (Norman, 1980; Whitehead et al, 2004). Depending on its position in space, a single leaf is affected differently by the environment. With advances in phenomics technologies, plant architecture can be precisely measured (Paprocki et al, 2012), represented and coded *in-silico* at the single plant level (Godin 2000) and/or at the canopy level (Deery et al, 2014). Repetitive reconstruction over time allows monitoring of plant or canopy growth non-destructively, thus enabling linkage of modelled plant carbon gains to real plant growth. With the emergence of high throughput non-invasive phenotyping platforms and easily accessible high performance computing clusters, 'Functional Structural Plant Models' or FSPM (Hanan, 1997; Godin and Sinoquet, 2005) represent a real opportunity for scaling photosynthesis and plant growth processes from both a structural and functional point of view. In these models, 3D architectural information is implicit because the spatial structure of the plant is deemed an essential factor contributing to its behaviour in response to the environment (Vos et al., 2007). Coupled to radiation

transfer models which allow prediction of the effects of incoming radiation at any point in a plant canopy (e.g. the radiative transfer model DART; Gastellu-Etchegorry, 1996), the heterogeneity in the thermal, aerodynamic and light climates within the canopy can be quantified and the properties of leaves, which varies in space and in time, can be taken into account. In fact, new computational techniques associated with new sensing technologies (e.g. far-infrared sensors) are allowing the mapping of photosynthetic functional properties onto leaves/canopies on 3-D models and this is opening new horizons (Sirault et al, 2013) in understanding comprehensively the consequences of the modifications discussed herein. It is now clear that advances in signal and data integration are going to become central in improving photosynthesis in cereal crops both by traditional phenotype driven breeding and through candidate gene transgenic modification.

Future Prospects

Photosynthesis is indeed one of the most intensely studied of all biochemical processes in terrestrial organisms, as one would expect of a pathway which underpins all life on earth. After 50 or more years of photosynthesis research we have only just begun to utilise this knowledge for engineering in crops. Yet there is so far no convincing demonstration of a positive effect of manipulating levels of photosynthetic proteins in cereals on photosynthetic performance or yield. The delay in applying our photosynthetic knowledge at the biochemical and molecular level may in part be due to the view that the proven green revolution route of pursuing grain number and harvest index would continue to deliver improvements in yield potential through traditional plant breeding. Successes in photosynthetic engineering in crops in the field are thus far limited to demonstrations that improved RuBP regeneration in tobacco by overexpression of SBPase has a positive benefit on photosynthesis, growth and yield in the field but this is very dependent on environmental conditions, having the largest benefit under elevated CO₂ (Rosenthal et al 2011). There are literature reports of the introduction of other single transgenes encoding enzymes involved in C₄ photosynthesis (such as PEP carboxylase) giving positive benefits on photosynthesis and yield in cereals (Ku et al 1999;), but these reports have been difficult to replicate and these genes have not so far been deployed in breeding any commercial cultivars. Improvement of Rubisco and introduction of a C₄-like or cyanobacterial CO₂ concentrating mechanism to C₃ cereals may all be more than a decade or more away. However, given the substantive knowledge base we have in photosynthetic mechanisms, some optimism is justified that we will at least produce prototypes which can be improved and fine-tuned within that timeframe. In the interim, physiological breeding programs have a wealth of new tools amenable to non-destructively and rapidly phenotype photosynthesis, growth and biomass (see Furbank and Tester 2011; Parry et al 2011; Deery et al 2014).

The future of photosynthetic engineering in crops may reflect the observations made in attempts to deploy marker assisted breeding and genomic selection: quantitative traits are often multigenic, the genetics are complex and plasticity and environmental components are confounding. Molecular physiologists can now modify entire pathways, introducing a suite of genes in single gene constructs using a variety of new molecular tools made possible through synthetic biology (eg Golden Braid; Sarrion-Perdigones et al 2011) but the “trait value” of these genes must be determined for rational design of approaches. Also, transgenic traits must be evaluated in the context of their cost effectiveness in crop breeding in regard to the existence of defined natural variation in the trait as genetic manipulation is not necessarily a low cost solution. Deployment of traits and genes from other species and organisms is a powerful tool for quantum changes in plant performance, but transgenic breeding may not be necessary if existing genetic variation can be effectively mined within a species or through wide crossing. Targeted manipulation of photosynthetic proteins in a meaningful way to produce a quantum improvement in yield potential must be guided by sophisticated modelling to identify appropriate gene targets and predict trait value based on sound crop physiology, not single pot experiments with model species. There are several obstacles to achieving such targeted improvement of photosynthetic performance, one of which is the lack of promoters with well defined temporal, tissue and cell specificity for cereal genetic transformation. Even for the less ambitious goal of altering amounts of photosynthetic proteins in different classes of leaves which are sunlit or shaded to optimise performance requires promoters which are not currently available, as does alteration of genes involved in leaf angle.

While manipulating the “engine of plant life” has large potential payoffs, cereal breeders of the future may have to wait more than a decade until they can deploy a range of these transgenic traits for given target environments in the same way as they do now with marker assisted selection.

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Figure 1

Figure 2

Figure 3

Figure 4