

Accelerating yield potential in soybean: potential targets for biotechnological improvement

ELIZABETH A. AINSWORTH^{1,2}, CRAIG R. YENDREK¹, JEFFREY A. SKONECZKA¹ & STEPHEN P. LONG²

¹USDA ARS Global Change and Photosynthesis Research Unit, 1201 W. Gregory Drive, Urbana, IL 61801, USA,

²Departments of Plant Biology and Crop Science, University of Illinois, Urbana-Champaign, 1201 W. Gregory Drive, Urbana, IL 61801, USA

ABSTRACT

Soybean (*Glycine max* Merr.) is the world's most widely grown legume and provides an important source of protein and oil. Global soybean production and yield per hectare increased steadily over the past century with improved agronomy and development of cultivars suited to a wide range of latitudes. In order to meet the needs of a growing world population without unsustainable expansion of the land area devoted to this crop, yield must increase at a faster rate than at present. Here, the historical basis for the yield gains realized in the past 90 years are examined together with potential metabolic targets for achieving further improvements in yield potential. These targets include improving photosynthetic efficiency, optimizing delivery and utilization of carbon, more efficient nitrogen fixation and altering flower initiation and abortion. Optimization of investment in photosynthetic enzymes, bypassing photorespiratory metabolism, engineering the electron transport chain and engineering a faster recovery from the photoprotected state are different strategies to improve photosynthesis in soybean. These potential improvements in photosynthetic carbon gain will need to be matched by increased carbon and nitrogen transport to developing soybean pods and seeds in order to maximize the benefit. Better understanding of control of carbon and nitrogen transport along with improved knowledge of the regulation of flower initiation and abortion will be needed to optimize sink capacity in soybean. Although few single targets are likely to deliver a quantum leap in yields, biotechnological advances in molecular breeding techniques that allow for alteration of the soybean genome and transcriptome promise significant yield gains.

Key-words: *Glycine max*; genetic engineering; photorespiration; photosynthetic efficiency; sink–source relations.

INTRODUCTION

At the 2008 World Food Security conference, United Nations Secretary-General Ban Ki-moon called for a 50% increase

Correspondence: E.A. Ainsworth, 1201 W. Gregory Drive, Urbana, IL 61801, USA. Fax: +(217) 244 4419; e-mail: lisa.ainsworth@ars.usda.gov

in global food production by 2030 in order to meet the increasing demand of a growing world population. Soybean is a key component of global food security, providing high-protein animal feed and over half of the world's oilseed production [United States Department of Agriculture (USDA) Foreign Agricultural Service's Production, Supply and Distribution database]. In terms of mass of seed produced, soybean is the fourth most important crop in the world and ranks second in the United States in terms of land area planted (FAOSTAT 2010; <http://faostat.fao.org/default.aspx>). Future demand for soybean will increase not only as the world population size increases, but also as incomes improve and diets become more meat-intensive. For example, in 1990 China's net imports of soybean were 1 Tg. This rose to 33 Tg by 2007 with the growth of its economy and a more than doubling of its national meat production (FAOSTAT 2010; <http://faostat.fao.org/default.aspx>).

The current global production of soybean is over 255 000 Tg, and just seven countries provide over 95% of global production (Fig. 1). Although the crop was first domesticated in China and was absent from the New World before European settlement, today 80% of the world's soybeans are grown in just three New World countries, the United States, Brazil and Argentina. Soybean yields in these three countries have increased steadily over the past two decades (Fig. 2a). The increasing yield trend, coupled with the dramatic increase in soybean acreage (Fig. 2b), has roughly doubled global soybean production since 1990 (Fig. 2c). From 1961 to 2007, approximately one-third of the increase in soybean production was attributed to increasing yields, whereas greater land area was responsible for the remaining two-thirds (Masuda & Goldsmith 2009). There is limited room for further expansion of the soybean production area in the United States, and there is social and political pressure to limit land use expansion in Brazil, where soybean cultivation is suggested to be one of the underlying direct and indirect causes of tropical deforestation (Barona *et al.* 2010). Therefore, in order to meet the increased demand expected for the coming decades without unsustainable expansion of the production area, soybean yields must be improved and at a more rapid rate than in the past.

What is the maximum yield that soybean might achieve? To date, there is little evidence that soybean yields are reaching a plateau (Fig. 2a; Egli 2008), yet the current rate

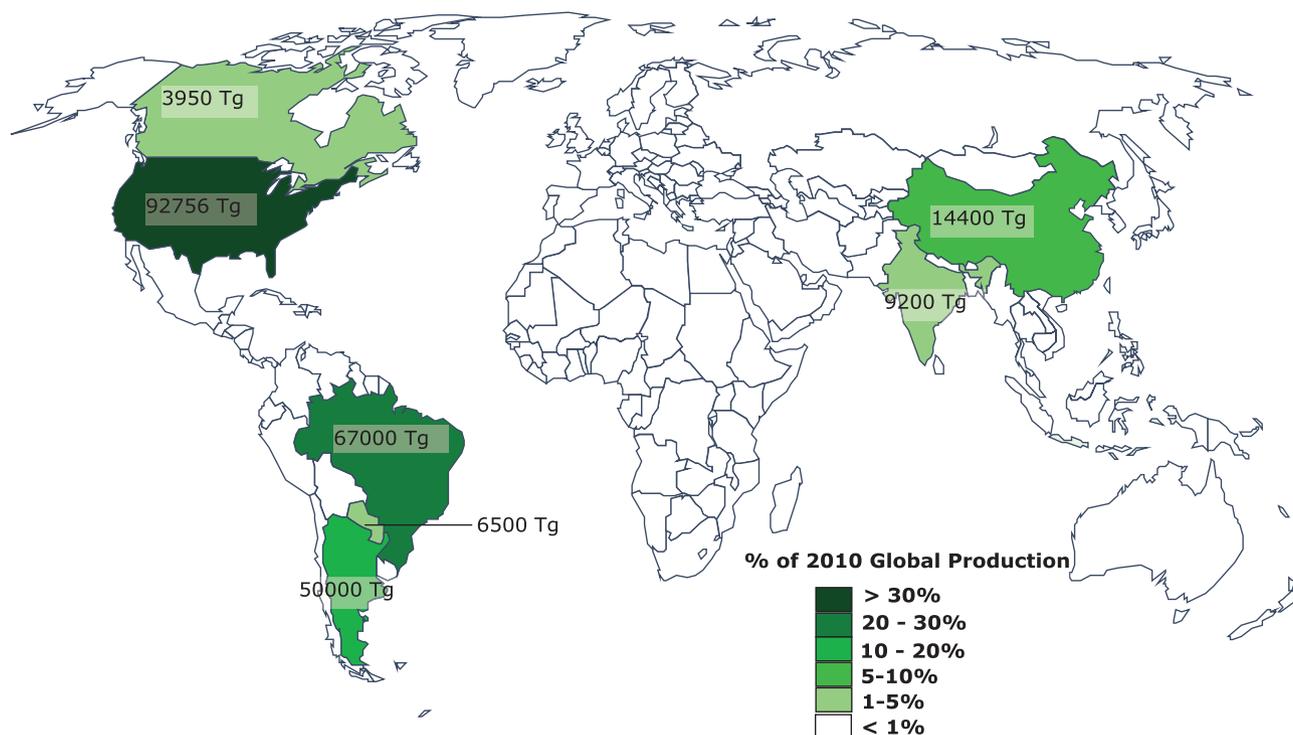


Figure 1. Percentage of global soybean production by nation in 2010. Country data were taken from the USDA Foreign Agricultural Service's Production, Supply and Distribution (PSD) online database (<http://www.fas.usda.gov/psdonline/psdHome.aspx>). Tg, Teragram = 1 million metric tons.

of increase is insufficient to meet the targeted 50% increase in production by 2030 without expansion of soybean production area. Yield, in any crop, is difficult to dissect as it is determined by a complex network of interactions of physiological, genetic, abiotic and biotic factors. Yield potential is defined as the maximum yield (seed dry matter) of a cultivar in an environment to which it is adapted, when grown with sufficient water and nutrients in the absence of abiotic and biotic stress (Evans & Fischer 1999). Although yield potential is difficult to measure accurately and varies from location to location, in 2010 the soybean yield world record was set at 10 760 kg ha⁻¹ in Missouri, USA (<http://mosoy.org/2010-yield-contest-release/>), and indicates that there is considerable opportunity to exploit the gap between average farm yields (<3000 kg ha⁻¹; Fig. 2a) and maximum, achievable yields. Therefore, efforts to improve both realized farm yields and yield potential in soybean are important, and improvements in yield potential will increase the speed and ease by which on-the-farm yield gains are attained in the future.

In this review, we first analyse the historical gains in soybean yields realized in the United States in the past 90 years. Secondly, we identify strategies for altering soybean metabolism, including improving photosynthetic efficiency and altering sink strength and metabolism. Thirdly, we discuss recent biotechnological advances for soybean germplasm enhancement. Although we also acknowledge that improving stress tolerance is important for maximizing soybean yields, this paper does not focus on biotic and

abiotic stress, but rather identifies potential biochemical and genetic targets for altering plant primary metabolism and carbon and nitrogen allocation. Strategies for engineering improved stress tolerance in soybean and other crops have been reviewed recently (see Valliyodan & Nguyen 2006; Phang, Shao & Lam 2008; Mittler & Blumwald 2010; Tran & Mochida 2010).

HISTORICAL PERSPECTIVE: ACHIEVING CURRENT YIELDS AND FUTURE POTENTIAL FOR ENHANCEMENT

In the ~90 years since soybean first became widely cultivated in the United States, production has been closely monitored by the USDA National Agricultural Statistics Service (NASS). Average yields in United States increased significantly from 740 kg ha⁻¹ in 1924 to 2986 kg ha⁻¹ in 2010 (Fig. 2a). This increase shows a strict adherence to a linear model ($R^2 = 0.94$), with annual gains of 22.2 kg ha⁻¹. Genetic developments, the release of new cultivars and improvements in farming technology have contributed to this continuous increase in soybean yield (Specht, Hume & Kumudinia 1999). This increase may also in part be a response to the increase in atmospheric CO₂ concentration ([CO₂]). Elevation of [CO₂] from 384 to 550 μmol mol⁻¹ under open-air field conditions caused soybean yield to increase by 15% (Long *et al.* 2006a). As the rate of yield increase for a given increase in [CO₂] is generally found to be less at higher concentrations (Long *et al.* 2004), it follows

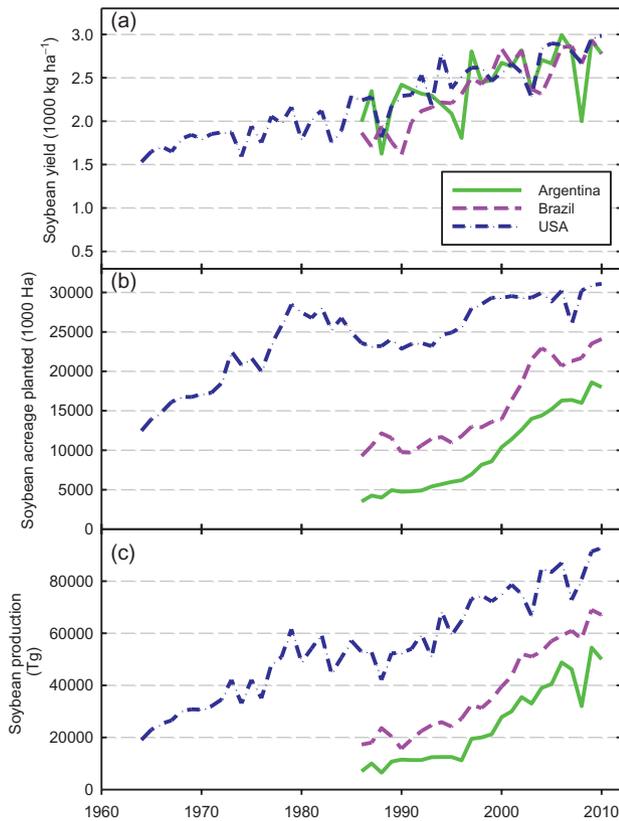


Figure 2. Historical changes in soybean yield (a), soybean acreage (b) and soybean production (c) in the United States, Brazil and Argentina. Country production data are from the USDA Foreign Agricultural Service's Production, Supply and Distribution (PSD) online database (<http://www.fas.usda.gov/psdonline/psdHome.aspx>).

that the minimum increase in yield resulting from the increase in $[\text{CO}_2]$ from $313 \mu\text{mol mol}^{-1}$ in 1924 to $392 \mu\text{mol mol}^{-1}$ in 2010 would be 7.1% or just under 0.1% per $\mu\text{mol mol}^{-1}$ increase. This, however, may have been offset in part by the yield depressing effect of the increase in surface ozone concentration, which is estimated to depress current yields by 15–20% (Morgan, Ainsworth & Long 2003; Morgan *et al.* 2006). Coincident with these overall yield gains has been the development of North American maturity groups (MGs) ranging from 000, suited to the shortest growing seasons in Canada, through X with the longest growing season for the southernmost of United States. Similar development of a range of MGs has been achieved in South American, extending to the tropical regions of Brazil (Alliprandini *et al.* 2009).

From 2001–2010, the rise in soybean yield per year increased more sharply to an average 44 kg ha^{-1} , based on a linear regression of average USA yields during that decade (data from USDA NASS; <http://www.nass.usda.gov>). An important development in soybean production that preceded this time period was the release of commercial, herbicide-tolerant (HT) or so-called 'round-up ready' soybean cultivars in 1996. Planted acreage of HT cultivars, predominantly those resistant to the herbicide glyphosate,

has increased steadily since and accounted for over 90% of the nationally planted soybean area by 2007 (data from USDA ERS; <http://www.ers.usda.gov>). The yield advantages of HT cultivars are clearly demonstrated by their rapid and wide adoption by farmers and their performance in independent variety trials on State Agricultural Experimental Stations (Fig. 3). For example, in the 2010 Illinois state variety trials, side-by-side yield trials of public, non-genetically modified organism (GMO) commercial and HT commercial cultivars revealed that HT commercial lines yielded significantly more than non-GMO commercial lines and public lines (Fig. 3). While the variance indicates that some public lines and non-GMO commercial lines can be competitive with HT commercial lines, the results indicate that average HT cultivar yields are higher. Increased performance of HT lines may result from both more efficient weed control in HT plots and more aggressive breeding efforts to establish elite germplasm with the HT trait. It is perhaps not surprising that of the 374 soybean cultivars registered from 2005 to 2008, 87% were glyphosate tolerant (Mikel *et al.* 2010).

In North America, initial development of cultivars for northern and southern regions proceeded more or less independently of each other (Gizlice, Carter & Burton 1993), although this divergence was from a narrow genetic base. In fact 75% of the genes found in cultivars released between 1947 and 1988 could be traced to just 17 early introductions (Gizlice, Carter & Burton 1994). Such a dilute pool of alleles, stemming from the continued use of elite lines developed downstream of a genetic bottleneck, would be expected to limit future genetic gains of soybean.

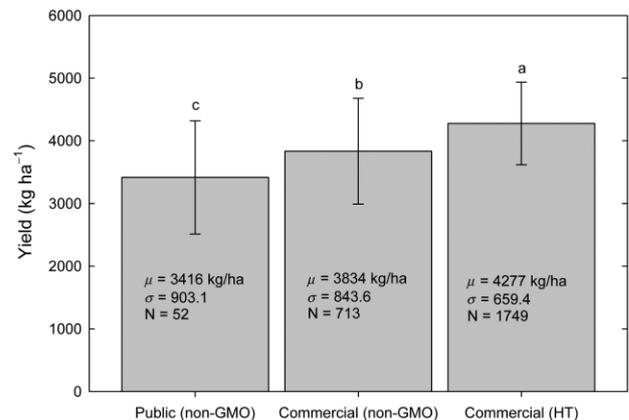


Figure 3. Comparison of mean yield (kg ha^{-1}) between public [all non-genetically modified organism (GMO) cultivars], non-GMO commercial cultivars and herbicide tolerant (HT) commercial cultivars. Means were calculated from data collected during the 2010 Illinois Variety Trial, which included 169 conventional and 419 HT varieties from 42 seed companies, grown across 13 locations in Illinois. Error bars represent the standard deviation to better demonstrate the variance observed for each group. All means are significantly different from each other ($P < 0.001$). Data was obtained from the Varietal Information Program for Soybeans website (<http://www.vipsoybeans.org>).

There has been some effort to bridge the gap between northern and southern gene pools, which has likely contributed to increased genetic diversity in the past two decades (Sneller 1994). Genetic diversity in soybean has also been increased by the use of exotic germplasm in breeding programs, such as foreign elite lines or diverse plant introductions (PIs; for more information on PIs, and the USDA National Plant Germplasm System, see <http://www.ars-grin.gov/npgs>). Although the need to incorporate more diverse germplasm into modern cultivars is not a recent idea (Thorne & Fehr 1970; Schoener & Fehr 1979; Thompson & Nelson 1998), early attempts to use exotics were hindered by linkage between favourable and undesirable alleles embedded in the adjacent chromosomal regions. Several studies have shown that although high yielding lines can be produced from crosses between domestic and exotic lines, yield is typically inversely proportional to the percent of PI parentage (Schoener & Fehr 1979; Vello, Fehr & Bahrenfus 1984; Ininda *et al.* 1996). However, most of these observations were made after relatively few rounds of selection. Elite lines have undergone considerably more selection cycles to rogue out deleterious alleles and fine tune epistatic interactions that affect desirable traits. Molecular marker technology has also made it easier to circumvent linkage drag, accelerating the effectiveness of incorporating diverse germplasm.

Soybean breeders have identified numerous quantitative trait loci (QTL) associated with plant yield (Orf *et al.* 1999; Yuan *et al.* 2002; Kabelka *et al.* 2004; Guzman *et al.* 2007; Palomeque *et al.* 2009). Many of these yield QTL owe their high-yielding allele to an exotic or PI parent. However, the vast majority of the QTL identified in such studies have not been introgressed into cultivars or selected for in ongoing breeding programs. The likely reason for this stems from questions regarding their validity. QTL for low heritability, highly polygenic traits such as yield are notoriously susceptible to environmental effects, and can therefore be masked by external factors. Still, Concibido *et al.* (2003) identified a significant yield QTL in a *Glycine soja* introduction, confirmed its effect in back-cross lines, then introgressed the QTL into elite *G. max* germplasm. The net result was a 9% yield advantage in individuals with the introgressed *G. soja* allele. This demonstrates the potential for using QTL to improve yields, yet it is important that reported QTL be confirmed, or better characterized down to their primary genetic component, so that their potential for application in breeding programs can be accurately assessed.

Recently, a powerful resource to aid in the validation and characterization of QTL has become available in the soybean whole genome sequence (Schmutz *et al.* 2010). Since the first public release of its draft sequence in 2008 (<http://www.phytozome.net>), it has allowed a sequence-based approach identifying seed quality QTL that have now been characterized and/or cloned (Maroof *et al.* 2009; Skoneczka *et al.* 2009; Bolon *et al.* 2010). Additionally, it has aided in the identification of candidate genes for a number of insect and disease resistance genes (Meyer *et al.* 2009;

Kim *et al.* 2010). Validation of these candidate genes can now be more easily achieved through reverse genetics approaches such as gene silencing or insertional mutagenesis. Many of these studies utilized a fine-mapping strategy to shorten the QTL interval prior to identification of candidate genes, an approach that could now be used to identify yield-related QTLs. The identification of a yield QTL's causal genetic entity would be a significant, practical achievement, and would begin to clarify the contributors to this highly polygenic trait. However, from a breeding standpoint, it is perhaps more practical to focus on what identified yield QTL regions can contribute through introgression into new cultivars. A recent trend in plant breeding is that of genome-wide selection, a process that uses dense, genome-wide linkage maps to quantify a genetic value for an individual (Meuwissen, Hayes & Goddard 2001). Because of its comprehensive approach, it is being proposed as a more effective selection tool than conventional marker-assisted selection (Bernardo & Yu 2007). The recent re-sequencing of 17 *G. soja* and 14 *G. max* cultivars revealed 205 614 SNPs (Lam *et al.* 2010), showing that the necessary molecular marker framework exists to utilize a genome-wide approach to selection. Sequence comparisons suggest that *G. soja*, the wild progenitor of the allotetraploid *G. max*, represents a substantially different germplasm pool. *G. soja* also occupies a much wider geographic range, than the assumed region of domestication on the Yellow River suggesting that there remains a large unexploited germplasm pool for improvement via molecular breeding tools (Li *et al.* 2010).

Along with understanding the genetic changes that have enabled current soybean yields, it is also informative to investigate the physiological contributions to the historical gain in yields in order to identify potential targets for future improvements. Historical analyses of cultivars released throughout the past 90 years indicate that a major driver of the increase in yields is an increase in seed number per plant (Morrison, Voldeng & Cober 2000; Jin *et al.* 2010). However, there has not been any change in individual seed weight over time. Photosynthetic rates and harvest index have also increased in more modern cultivars, whereas leaf area index (LAI) has decreased (Morrison *et al.* 2000; Jin *et al.* 2010). Perhaps because of lower LAI, more recently released cultivars have improved performance in high plant density compared with older cultivars (Cober *et al.* 2005). Additionally, newer cultivars in both North America and China have decreased height and increased resistance to lodging (Wilcox *et al.* 1979; Morrison *et al.* 2000; Jin *et al.* 2010). Both of these changes might be expected as inadvertent results of selection for increased production. In nature there is strong selective pressure at the level of the individual for shading competitors, which can be achieved by gaining height and a leaf area supra-optimal for productivity. Excess leaf area is also an insurance against defoliating events, such as insect attacks and weather damage. In a well-managed monoculture, both of these characters of importance to natural selection will be deleterious to productivity.

TARGETS FOR ALTERING SOYBEAN METABOLISM THAT HOLD THE POTENTIAL FOR YIELD ENHANCEMENT

Improving soybean photosynthetic efficiency

The historical positive correlation between photosynthesis and yield in soybean suggests that improving photosynthetic efficiency might be a promising target for further yield gains. As defined by Monteith (1977), yield of a crop at any given location is the product of the incident photosynthetically active radiation, and the efficiencies with which it intercepted (ϵ_i), the intercepted PAR is converted into biomass (ϵ_c), and the efficiency with which the biomass is partitioned into seed (ϵ_p), also termed harvest index (Zhu, Long & Ort 2010). It has been argued that ϵ_i and ϵ_p have been maximized for modern crops (Long *et al.* 2006b; Murchie, Pinto & Horton 2009; Zhu *et al.* 2010), including soybean. The canopy of a modern cultivar of soybean growing in central Illinois was shown to intercept ~90% ($\epsilon_i = 0.9$), of the incident PAR integrated over the growing season, and to partition ~60% ($\epsilon_p = 0.6$) of the biomass energy into seed (Zhu *et al.* 2010). These achievements in ϵ_i and ϵ_p seem to leave little room for further improvement in soybean. As ϵ_i represents the interception efficiency over the growing season, greater yield may be obtainable if at a given location the growing season could be extended. Growing seasons in the corn belt of the United States are generally limited by temperature, and in the western United States by moisture. Identification of germplasm capable of development and maintenance of leaves at lower temperature or lower water potentials could allow breeding of more productive lines, as could an improved understanding of the gene networks affecting these characters. In the absence of growing season extension, ϵ_c remains an important, and mathematically perhaps the only remaining target for improvement of yield potential.

Recent estimates of maximum theoretical ϵ_c for soybean and other C₃ plants range from 4.1 to 4.6%, at current [CO₂] and 30 °C (Zhu, Long & Ort 2008; Amthor 2010). Soybean grown in productive soils in central Illinois achieved ϵ_c of 1.6% at an atmospheric CO₂ concentration of 380 ppm (Zhu *et al.* 2010), falling well short of the theoretical maximum. The observation that neither ϵ_i nor ϵ_p increased while leaf photosynthesis, ϵ_c and seed yield increased in soybean exposed to season-long elevation of CO₂ concentration suggests that attempts to increase ϵ_c by altering photosynthetic metabolism could have similar beneficial effects on seed yield (Zhu *et al.* 2010). Targets for improving ϵ_c and enhancing C₃ photosynthesis have been the subject of a number of recent papers (Long *et al.* 2006b; Peterhansel, Niessen & Kebeish 2008; Zhu *et al.* 2008, 2010; Murchie *et al.* 2009; von Caemmerer & Evans 2010; Ort, Zhu & Melis 2011; Parry *et al.* 2011), and are collectively hypothesized to boost yield potential by up to 50% (Long *et al.* 2006b). In the following section, we will briefly review potential targets for improving ϵ_c , highlighting those with the greatest potential to be realized in soybean in the next 20 years.

A natural starting point for improving ϵ_c in C₃ plants is ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), the primary enzyme of CO₂ fixation, which is competitively inhibited by O₂ (Spreitzer & Salvucci 2002). One strategy for improving the performance of Rubisco is to alter its specificity for CO₂ relative to O₂ (Zhu, Portis & Long 2004b; von Caemmerer & Evans 2010). Although increasing the specificity of Rubisco would increase photosynthesis when ribulose-1,5-bisphosphate (RuBP) is limiting (von Caemmerer & Evans 2010), there is a trade-off between specificity and catalytic rate (Bainbridge *et al.* 1995; Zhu & Spreitzer 1996). The average specificity factor for C₃ crop canopies was modelled to exceed the optimal level for today's atmospheric carbon dioxide concentration ([CO₂]) (Zhu *et al.* 2004a). Therefore, an 'optimal canopy' might have a Rubisco with low specificity and high catalytic rate in the upper canopy, and high specificity, lower catalytic rate Rubisco in the lower canopy (Zhu *et al.* 2010). Rubisco enzymes with a range of specificities and catalytic capacities are found in naturally occurring photosynthetic organisms (Jordan & Ogren 1981, 1984; Parry, Keys & Gutteridge 1989; Galmes *et al.* 2005). However, *Limonium gibertii*, a plant adapted to a hot, arid environment, has significantly higher catalytic rates than average C₃ species, and also maintains a higher specificity (Galmes *et al.* 2005). This provides a potential model for avoiding the observed trade-off between specificity and catalytic capacity, and a tool for improving both properties in crops (Parry *et al.* 2007). It also suggests that more efficient Rubiscos are likely to be discovered. However, there are significant technical barriers to overcome before soybean can be efficiently transformed with foreign Rubisco or engineered by mutagenesis (Parry *et al.* 2007; Peterhansel *et al.* 2008). A major complexity is the need to replace both the plastid-encoded large subunit and the nuclear-encoded small subunit in order to ensure that an effective holoenzyme is expressed in the plastid. Foreign small and large subunits have been successfully engineered into tobacco (Whitney & Andrews 2001; Dhingra, Portis & Daniell 2004) and key steps controlling the assembly of the Rubisco holoenzyme were recently reported (Liu *et al.* 2010). Still, a 'better' Rubisco has yet to be engineered in higher plants (Whitney, Houtz & Alonso 2011). Recently, the genes encoding two forms of soybean Rubisco activase, which is key to the activation and stability of Rubisco, were cloned and characterized (Yin *et al.* 2010). Expression of these genes was positively correlated with Rubisco activity, photosynthetic rate and seed yield. Thus, altering Rubisco activase may provide another approach for enhancing soybean photosynthesis and productivity (Spreitzer & Salvucci 2002; Yin *et al.* 2010).

Although engineering improved Rubisco might be technically challenging in the short term, altering plant investment in other enzymes of primary metabolism is currently feasible. In particular, overexpression of sedoheptulose-1,7-bisphosphatase (SBPase) in tobacco increased photosynthesis and biomass production (Raines 2006). This remains to be tested in soybean in a field setting, but it is a promising target for enhancing photosynthesis. Using a

dynamic metabolic model of C metabolism, Zhu, de Sturler & Long (2007) found that the current partitioning of nitrogen among the enzymes of C₃ carbon metabolism was not optimized to today's atmospheric [CO₂]. The model predicted that SBPase should be increased, consistent with the experimental data, and also predicted that ADP glucose pyrophosphorylase could be increased, while photorespiratory enzymes could be decreased. In addition to optimizing concentration of specific Calvin cycle enzymes, increasing overall investment in photosynthetic proteins may also be beneficial. In *Arabidopsis*, investment in proteins involved in primary metabolism was positively correlated with biomass accumulation in 129 accessions (Sulpice *et al.* 2010). Therefore, it appears that increased investment in photosynthetic capacity is a potential strategy for at least increasing biomass and potentially improving seed yield in C₃ crops.

In soybean and other C₃ plants, the oxygenation reaction and subsequent photorespiration account for a significant loss of energy, and as mentioned previously C₃ crops appear to over-invest in photorespiratory enzymes (Zhu *et al.* 2007). Therefore engineering plants to reduce photorespiration would improve conversion efficiency. One strategy for reducing photorespiration is to engineer a CO₂ concentrating mechanism into plants (Edwards *et al.* 2001; Hibberd, Sheehy & Langdale 2008). CO₂ is a competitive inhibitor of the oxygenase reaction of Rubisco. The dicarboxylate cycle of C₄ photosynthesis serves as a light driven pump, concentrating CO₂ at Rubisco, to a sufficient level to largely eliminate photorespiration. One solution would therefore be to engineer Kranz anatomy and the C₄ pathway into soybean. However, this appears to require many changes, not only the expression of two photosynthetic tissue types in the place of one, but also expression of the C₄ and C₃ enzymes and transporters in the correct tissues and organelles. Given that the gene networks underlying the development of C₄ structure and function remain incompletely understood, such transformations were considered long-term goals (Zhu *et al.* 2010). However, an alternative viewpoint is that as C₄ photosynthesis is broadly similar across flowering plants, yet has evolved multiple times (Sage 2004), there may be relatively simple and conserved pathways that would facilitate a rapid conversion of C₃ to C₄ plants (Hibberd *et al.* 2008). Considerable effort is being invested in work towards engineering the C₄ syndrome into rice, and clearly if successful would indicate the path for converting other C₃ crops (Edwards *et al.* 2001; Hibberd *et al.* 2008).

In the shorter term, a strategy may be to engineer the CO₂ concentrating mechanism of cyanobacteria into soybean chloroplasts. For example, *Synechococcus* has membrane proteins that actively pump both bicarbonate and CO₂ into the photosynthetic cell. A further sophistication in some species is the presence of carboxysomes, an ordered structure that encloses Rubisco and carbonic anhydrase within a coat protein. This creates a local high concentration of CO₂ at the site of Rubisco where the conversion of bicarbonate to CO₂ is accelerated (Price,

Coleman & Badger 1992; Price *et al.* 1998; Badger, Hanson & Price 2002). Chloroplasts are considered to have evolved from ancestral cyanobacterial symbionts, which may have lost these concentrating mechanisms as plants evolved from carbon-limited aquatic systems to the assumed high CO₂ world of the first terrestrial plants. Given the relationship of plastids to cyanobacteria, re-introducing these prokaryotic genes may be feasible. Indeed, Lieman-Hurwitz *et al.* (2003) produced transgenic *Arabidopsis* and *Nicotiana tabacum* plants that expressed the *ictB* gene involved in bicarbonate accumulation in *Synechococcus*. These plants had significantly lower CO₂ compensation points of photosynthesis showing decreased photorespiration and significantly increased rates of leaf CO₂ uptake when CO₂ availability was limiting, but not when it was saturating. Given this success with two other dicotyledonous species, this would appear a promising target for improving soybean photosynthesis.

Another strategy for reducing photorespiration, and in particular the energy lost in the current photorespiratory pathway of soybean C₃ crops, is to express the key genes of one of the *Escherichia coli* pathways for the metabolism of glycolate to phosphoglycerate, which has been successfully done in *Arabidopsis* (Kebeish *et al.* 2007). Here, a three reaction pathway of conversion of two molecules of glycolate to one of phosphoglycerate was engineered into the chloroplast. The pathway bypasses the photorespiratory reactions normally involving the cytosol, peroxisomes and mitochondria, resulting in reduced metabolite flow through photorespiration, enhanced carbon assimilation and improved growth in transgenic plants (Kebeish *et al.* 2007; Peterhansel *et al.* 2008). Although this pathway still releases one molecule of CO₂ for every two molecules of glycolate formed, it has two advantages. Firstly, the CO₂ is released within the chloroplast, which more effectively increases CO₂ concentration around Rubisco. Secondly, no ammonia is released, which in normal higher plant C₂ metabolism requires a large amount of reductive power to re-assimilate. The pathway does produce NADH, which would lead to a nucleotide imbalance; however, based on parallel directed evolution of metabolic pathways in *E. coli*, modification to NADPH utilization is unlikely to represent a major barrier. To avoid parallel use of the native C₂ metabolic pathway, the plastid glycolate transporter would also need to be knocked-out. It has been suggested that photorespiration has a photoprotective role, particularly in young, expanding soybean leaves exposed to high light at the top of the canopy (Jiang *et al.* 2006). However, reduction of photorespiration by open-air elevation of CO₂ in the field was not found to cause any loss of photosystem II (PSII) operating efficiency, as an indicator of photoprotection or photoinhibition, at any stage in the plant life cycle (Rogers *et al.* 2004; Bernacchi *et al.* 2006). Further, as noted by Jiang *et al.* (2006), leaf movement and xanthophyll de-epoxidation also play key parts in protection. The xanthophyll cycle can be up-regulated, with apparently little additional investment, and as de-epoxidation is inducible, photosynthetic efficiency is only lowered under conditions of excess light. By

contrast, photorespiration will operate and impose inefficiency on net photosynthesis regardless of light conditions (Raven 1989; Zhu *et al.* 2004a)

Other potential opportunities for improving ϵ_c involve engineering changes to the photosynthetic electron transport chain (Peterhansel *et al.* 2008; Melis 2009; Murchie *et al.* 2009; Zhu *et al.* 2010). Plants appear to overinvest in chlorophyll associated with the photosystem core complexes, so engineering a smaller antenna in leaves at the top of the soybean canopy might mitigate efficiency losses associated with overexcitation and induction of non-photochemical quenching (Melis 2009; Ort *et al.* 2011; Zhu *et al.* 2010). Lower chlorophyll content in upper canopy leaves may also allow for higher concentrations of chlorophyll lower in the canopy, which would allow more efficient light harvesting in the light-limited environment. Chlorophyll-deficient soybean mutants with approximately half the chlorophyll content of wild-type showed increased daily photosynthetic gains (Pettigrew *et al.* 1989), providing experimental support for this suggestion. Another change to the light-harvesting apparatus that is hypothesized to improve ϵ_c is engineering a faster recovery from the photoprotective state (Zhu *et al.* 2004a; Zhu *et al.* 2010). PSII switches to a photoprotective state when there is excess light that cannot be used for photosynthesis, and the return to the high-efficiency state is very slow relative to the rapid fluctuations in the light environment of leaves within a canopy in the field. The cost of the slow recovery from the photoprotective state was modelled to be 15% of daily canopy carbon gain in typical temperate crops (Zhu *et al.* 2004a). Far more rapid recovery has been observed in nature, suggesting that faster recovery is possible (Zhu *et al.* 2004a). Therefore, engineering more rapid recovery from the photoprotected state could lead to very significant enhancements in photosynthesis, with potential for improvements in plant growth and yield.

Altering sink strength and metabolism

In addition to strategies to improve yield potential by improving photosynthesis, there may be potential to increase soybean yield by modifying source–sink relations to increase the sink strength of developing soybean pods. Defined as the ability to import photoassimilate into sink tissue, actual sink strength is a factor of net carbon gain via phloem transport minus respiratory carbon loss caused by growth and maintenance (Ho 1988). Evidence that modern soybean cultivars are sink limited comes from experiments performed at elevated $[\text{CO}_2]$. In these studies, leaf photosynthesis across the daylight hours and growing season was increased on average by 24% (Bernacchi *et al.* 2006), but yield of the same crop was only increased by 15%, and harvest index was also significantly decreased (Morgan *et al.* 2005). This result shows that control of yield is shared between both source and sink, and that increase in sink strength potential will be necessary to take full advantage of any increase in source activity, for example, net photosynthesis. It has become increasingly clear in recent years

that N assimilation and C metabolism are intricately coordinated by a complex network of metabolites, gene expression and enzyme activities (reviewed by Nunes-Nesi, Fernie & Stitt 2010). As soybean is a legume, this coordination also involves allocation of C to *Bradyrhizobium japonicum* in return for N. Strategies for modifying C and N allocation and sink strength potential can be organized into the following categories: (1) increasing C and N import into developing seeds; (2) maximizing respiratory efficiency by shifting the balance away from catabolism and towards anabolism; (3) increasing the number of pods by controlling reproductive development; and (4) utilizing an optimized *B. japonicum* strain that delivers more fixed N for less photosynthate.

Long-distance transport of sugar begins in the source leaves, where sucrose that was synthesized in the mesophyll is loaded into the phloem by members of the SUCROSE TRANSPORTER family (SUC or SUT), which are membrane-localized, energy dependent, H^+ -symporting proteins (reviewed in Lalonde, Wipf & Frommer 2004; Sauer 2007; Kuhn & Grof 2010). Once translocated to reproductive sinks, sucrose is unloaded from the seed coat into the apoplastic space between maternal and filial structures, where it is then taken up by the developing seeds (reviewed in Thorne 1985; Patrick 1997; Weber, Borisjuk & Wobus 2005; Zhang *et al.* 2007). When the sink-limited nature of soybean seed development was examined, the pre-storage phase was identified as the most responsive to changes in carbon supply (Borrás, Slafer & Otegui 2004). Therefore, efforts to increase sucrose transport during early pod development (stages R3–R6) may result in increased yield. To test the role of SUT activity in accumulating photoassimilates from the seed apoplasm, the potato *SUT1* was over-expressed in *Pisum sativum* (pea) cotyledon storage parenchyma cells using the seed-specific pea vicilin promoter (Rosche *et al.* 2002). These transgenic plants had increased rates of sucrose transport into cotyledons, higher cotyledon growth rates (Rosche *et al.* 2002), and increased storage protein levels (Rosche *et al.* 2005), but no change in yield was observed. This was likely because of the fact that *SUT1* overexpression was restricted to storage parenchyma cells within the cotyledon. This is downstream of transfer cells, which import sucrose released into the apoplasm by the seed coat and are essential for establishing strong sink strength (Weber *et al.* 2005). Identifying the most appropriate *SUT* gene (or combination of genes) localized along the source/sink pathway to manipulate sucrose import into developing soybean pods will be a challenge. However, feedback regulation studies suggest that increasing sink demand through enhanced phloem transport will not be limited by photosynthesis (Vaughn, Harrington & Bush 2002), and represent a feasible strategy to achieve yield increases.

A complementary strategy for increasing seed yield is to manipulate nitrogen transport. In soybean, nitrogen is transported to seeds in the form of ureides from the roots via xylem and in the form of amino acids from the leaves via phloem (Rentsch, Schmidt & Tegeder 2007). A clear role for

N transporters in determining storage protein levels and seed yield has been demonstrated by the amino acid transporter AAP1 (Sanders *et al.* 2009), which has been shown to be localized to seed endosperm and developing embryos in *Arabidopsis* (Hirner *et al.* 1998). Another member of this family, AAP2, is localized in the phloem throughout the plant and is thought to function in amino acid transfer between xylem and phloem (Zhang *et al.* 2010). Mutant analysis revealed the importance of AAP2 in providing N supply to the photosynthetic apparatus, which affects development of source tissue, as well as C export and sink development. In addition to these amino acid transporters, plants have a large number of functionally uncharacterized peptide and nitrate transporters, many of which are localized to seeds (Tsay *et al.* 2007), and represent targets for manipulation.

Improved understanding of the complex, interconnected relationship between whole-plant C and N status with photosynthesis, source/sink balance and growth is identifying new potential targets for improving plant productivity and seed yield (Paul & Foyer 2001; Smith & Stitt 2007; Nunes-Nesi *et al.* 2010). For example, there is recent evidence that enhanced cytosolic pyruvate, orthophosphate dikinase (PPDK) levels lead to faster nitrogen export from senescing leaves, increased plant growth, increased seed weight and higher N content in *Arabidopsis* and *N. tabacum* (Taylor *et al.* 2010). The authors propose that PPDK functions in concert with a portion of the TCA cycle to produce the transport amino acid glutamine (Taylor *et al.* 2010). However, seed weight per plant remained constant in plants over-expressing PPDK, suggesting compensatory responses between seed number and individual seed weight. In soybean, there is a negative correlation between yield and protein concentration (e.g. Carter, Burton & Brim 1982; Rotundo *et al.* 2009), so it will be important to test if PPDK over-expression in soybean could lead to higher protein seeds in a high yielding genetic background.

Improvement of carbon balance has traditionally been approached by either increasing photosynthesis or reducing respiration rates (Gifford *et al.* 1984). However, the anabolic reactions of photosynthesis are balanced by catabolic reactions requiring photosynthate. The interconnected coordination of photosynthetic and respiratory metabolism in soybean can be seen in the context of atmospheric change. Elevated [CO₂] stimulates CO₂ assimilation, sugar and starch production, and leads to transcriptional reprogramming of respiratory genes, stimulating respiration rates of soybeans grown in the field (Davey *et al.* 2004; Leakey *et al.* 2009). Beneficial effects of mitochondrial oxidative metabolism during photosynthesis (in the light), including protection against photoinhibition, dissipation of redox equivalents exported from the chloroplasts (Raghavendra & Padmasree 2003), and supply of ATP for the Calvin cycle reactions (Nunes-Nesi *et al.* 2010), indicate that respiratory activity is essential for optimal photosynthesis. However, genetic manipulations of components of respiratory metabolism suggest that efficiency can be improved. For example, an aconitase mutant in tomato is characterized by

reduced TCA cycle metabolites and increased photosynthetic rates, sucrose synthesis and fruit yield (Carrari *et al.* 2003). Because of the reduced flux through the TCA cycle, an increase in accumulated sucrose in the source leaves was available for photoassimilate transport to the developing sinks, resulting in greater yields. A second strategy for modifying flux through the TCA cycle is seed specific repression of pyruvate dehydrogenase kinase. This enzyme is a negative regulator of the TCA cycle, and when repressed in *Arabidopsis* seeds, resulted in increased seed weight and seed oil content (Marillia *et al.* 2003), presumably by increasing sink strength via targeted increase in respiration within reproductive tissue. Whether or not using these strategies in field conditions will lead to higher soybean yields remains to be determined.

Soybeans include both determinate and indeterminate cultivars, and variations in those extremes. It might be expected that conversion to indeterminate would increase sink strength potential and yield. However, when a determinate cultivar and an isogenic line of the same cultivar with a single mutation making it indeterminate were grown side-by-side under elevated CO₂ concentration, the increase in photosynthetic CO₂ uptake at elevated CO₂ was not significantly greater in the indeterminate mutant (Ainsworth *et al.* 2004). This suggests that capacity to produce flower initials may not be limiting to sink strength potential, but development of initials into fertile flowers likely poses a limitation. Abortion rates of flowers in soybean can exceed 75% (vanSchaik & Probst 1958), despite the apparent ability, based on non-structural carbohydrate content, to support higher reproductive load (Streeter & Jeffers 1979). Understanding the underlying mechanisms controlling reproductive abortion, therefore, represents a potential way for crop improvement strategies to increase efficiency of carbon utilization. The influence of photosynthate supply on pod and seed number is evident from studies of irradiance (Schou, Jeffers & Streeter 1978), sucrose supplementation (Abdin *et al.* 1998), shading (Egli & Yu 1991) and defoliation (Board & Tan 1995). This relationship is presumed to be governed by photosynthesis, as sucrose export rates from leaves are positively correlated with net assimilation rates (Huber, Rogers & Mowry 1984).

Egli & Bruening (2002) hypothesized that competition for assimilate from early developing pods is a main factor in flower and pod abortion. This is based on their findings that flowering in soybean follows a bi-modal distribution, with 100% pod survival in the first cohort and <60% in the second, which is consistent with previous reports (Huff & Dybing 1980; Spollen, Wiebold & Glenn 1986). The asynchronous and extended duration (>30 days) of flowering in soybean was identified as a possible cause of late-flower abortion, as the large sink strength of fast-growing pods that were initiated early would demand preference for available photoassimilate and lead to shedding of less-developed pods. Although the mechanisms explaining this hypothesis are unknown, finding a way to synchronize floral initiation may promote greater pod survival. One possibility for manipulating flowering is to enhance the photoperiodic

signal that is perceived by the plant, with the intended goal of triggering floral initiation in a more coordinated manner. Much progress has been made in understanding photoperiodic flowering in soybean, and major roles for FLOWERING LOCUS T (FT; Kong *et al.* 2010) and CRYPTOCHROME 1 (CRY1; Zhang *et al.* 2008) have been identified. Therefore, using these targets to manipulate reproductive development may provide a way to synchronize flowering and in turn increase pod survival. In higher latitudes, early spring planting has been associated with maximum yields (Cooper 2003). Therefore, initiating flowering early, even in years when early planting is not possible may maximize potential yields by coordinating pod and seed development with the peak of radiation availability.

Rhizobial-mediated N fixation is an expensive undertaking from the perspective of the host plant, requiring 16 ATP equivalents for every N fixed (Dixon & Kahn 2004). Biological N fixation can supply up to 300 kg ha⁻¹ (Keyser & Li 1992), which is between 50 and 60% of total soybean N demand (Salvagiotti *et al.* 2008). Considering this gap, efforts have been made to explore the use of fertilizer to increase soybean yield potential. Although success has been made by timing the application of fertilization to coincide with pod filling (stages R3–R5; Wesley *et al.* 1998), questions remain on the cost-effectiveness and ecological impact of late-season fertilization. From an agricultural perspective, therefore, it is important to identify exploitable aspects of the soybean–*Rhizobium* symbiosis that could lead to increased N fixation efficiency. One possibility is to investigate host–symbiont compatibility, in particular, the ability of the host to withhold O₂ in response to how much N₂ is being fixed by the symbiont (Kiers *et al.* 2003). These so-called host sanctions are thought to provide a selective environment that promotes cooperation between plant and *Rhizobia*. Despite this, however, modern soybean cultivars are unable to limit nodulation by less-affective *Rhizobial* strains as successfully as older cultivars (Kiers, Hutton & Denison 2007). Therefore, it seems realistic to expect that improvements could be made in optimizing the efficiency of the host–symbiont relationship by taking a closer look at the effect that artificial selection via domestication and breeding of soybean has had on nodulation. Although this will not directly increase sink strength in developing pods, maximizing biological N fixation efficiency will result in making more photoassimilate available for transport into reproductive development.

USING ADVANCED BIOTECHNOLOGY APPLICATIONS TO ENGINEER ENHANCED YIELD IN SOYBEAN

Just as new technologies are enabling a more robust analysis of gene expression, for example using the soybean genome along with next generation sequencing techniques to quantify global changes in transcript abundance (Libault *et al.* 2010; Severin *et al.* 2011), molecular breeders must strive to adopt biotechnological advances that facilitate the incorporation of putative yield enhancement genes in order

to create new elite germplasm. Achieving this goal will consist of developing new tools as well as applying new insights/discoveries for breeding for yield enhancement.

Advanced crop transformation strategies exemplify new tools that are available to molecular breeders. The percent of all genetically modified soybeans grown in the United States reached 93% in 2010 (NASS Acreage Report 2010; <http://www.nass.usda.gov>), with the majority of these consisting of single-gene transgenes that confer herbicide-resistance. However, since the introduction of herbicide-resistant soybean in 1996, significant populations of weed species have become resistant to glyphosate (Powles 2008). Because of this phenomenon, it will be necessary for additional herbicide tolerance genes to be identified and transformed into elite germplasm on a perpetual basis. Further, in order to incorporate the potential yield enhancement targets described in this article and as new biotic and abiotic stress resistance as well as quality traits are identified, the number of genes needed to be introduced will continue to rise. Considering the time involved with moving GMOs through the regulatory process, single-gene transformations will not be able to keep pace with gene discovery, rendering this technology obsolete.

In response to these limitations, the industry has begun to move towards gene-stacking techniques. Gene stacking is based on mini-chromosome technology, which is essentially done by isolating species-specific centromeric DNA and introducing it back into plant cells by particle bombardment (Carlson *et al.* 2007). The introduced DNA is recognized by the cell as an endogenous chromosome and is faithfully replicated through meiotic and mitotic divisions; stability has been demonstrated for up to 10 generations. This technique has several attractive features, namely that the synthetic chromosome can be engineered with large amounts of DNA, allowing several genes to be incorporated, or stacked, into the host genome with a single transformation event. In addition, because the introduced DNA is not incorporated into the host genome, there will not be any positional effects that alter expression of the transgene, nor will there be any pleiotropic effects caused by random insertion that could disrupt genes crucial for metabolism or development. Currently, this technology, which was developed by Chromatin, Inc., is being licensed for use by Monsanto, Syngenta, Dow Agrosience and Bayer. Chromatin also has an exclusive technology combination agreement with Dow to be able to modify existing synthetic chromosomes using Dow's zinc finger nuclease technology (Cai *et al.* 2009). The versatility of these combined technologies should result in a shorter development timeline, making it possible to move new traits from the lab to the field much faster than the current 12–15 years.

Another tool for crop improvement adapts microRNA technology for molecular breeding applications. Specifically, artificial microRNAs (amiRNAs) can be designed using miRNA precursor backbones modified to incorporate target sequence from a yield enhancement gene of interest. Once transcribed, the synthetic pri-amiRNA is processed normally, allowing the mature amiRNAs to mediate the

degradation of target mRNA by forming double stranded RNA, that is subsequently recognized by ARGONAUTE (AGO1) and the silencing complex (reviewed in Liu & Chen 2010). The most obvious use of amiRNA technology is to augment the plant's natural defence system by eliminating mRNA sequences introduced by viruses (Duan *et al.* 2008). However, it could also be used for yield enhancement by custom silencing aspects of metabolic pathways determined not to be critical for yield. Furthermore, as more information is obtained about the targets of miRNA-mediated regulation, it will become desirable to block the function of a specific miRNA. Called target mimicry, this strategy aims to sequester an endogenous miRNA species, thereby preventing degradation of an mRNA that would normally be cleaved. In addition to the many transcription factors that are miRNA targets, two photosynthetic targets include the Calvin cycle genes coding for the transketolase that is targeted by gma-miR1530, and Rubisco, targeted by gma-miR1536 (Song *et al.* 2011).

In terms of linking discovery with practical applications, there is potential for incorporating new insights about plant development and metabolism from model plants for use in engineering yield enhancement in soybean. An exciting possibility is to manipulate transcription factors that have been shown – primarily from studies in *A. thaliana* – to be involved with the molecular regulation of agronomically important traits, including yield (Gonzalez, Beemster & Inzé 2009). Ongoing efforts to untangle the network of transcriptional control over the transition from vegetative to reproductive growth have identified master regulatory gene(s) that if manipulated, may induce the formation of additional flowers and pods. Of particular interest is the MADS-box transcription factor APETALA1 (API), which is involved with establishing floral meristem identity, and the bZIP transcription factor FLOWERING LOCUS D (FD), which along with the mobile protein FT is responsible for floral initiation (reviewed in Kaufmann, Pajoro & Angelet 2010). Because these transcription factors are key players in regulating the global flowering network, it may be possible to increase flower number by manipulating transcription within the inflorescence and floral meristems using these genes as targets for genetic engineering.

A second approach using transcription factors to engineer enhanced yield would be to induce a coordinated up-regulation of photosynthetic genes in the chloroplast with the goal of increasing the abundance of photosynthetic machinery on the thylakoid membrane, and by extension, increasing CO₂ assimilation. Redox status of the plastoquinone pool between photosystems is monitored by CHLOROPLAST SENSOR KINASE (CSK), which interacts functionally with PLASTID TRANSCRIPTION KINASE (PTK) and SIGMA FACTOR-1 (SIG-1) to comprise a signal transduction pathway that adjusts photosystem stoichiometry within the chloroplast via transcriptional regulation (Puthiyaveetil *et al.* 2008; Puthiyaveetil *et al.* 2010). Because this regulation serves to acclimate leaves to light quality and quantity, it may also be possible to engineer higher photosynthetic rates by altering light

perception through the manipulation of photoreceptors with the intended goal of generating crops with enhanced sun-leaf traits. Similar to the way transcription factors regulate downstream networks, modifying light signalling at the point of perception would likely affect light-dependent aspects of chloroplast and leaf development, as well as metabolism. Evidence to support this idea comes from microarray data that links irradiance sensing with CRY1 (Kleine *et al.* 2007) as well as the fact that light signalling through CRY1 is critical for normal chloroplast biogenesis (Ruckle, DeMarco & Larkin 2007). Like most attempts to modify plant metabolism, if confirmed, this strategy would lead to undesirable effects from a yield perspective, such as increased respiratory C loss needed for increased production and turnover of photosynthetic machinery. However, if improvements to photosynthetic efficiency and/or capacity can be coupled with more efficient respiration and increased sink strength, major increases in yield may be realized.

CONCLUSION

Soybean breeders and agronomists have produced steady yield gains over the past 50 years (Fig. 2). While there may be room for adding useful genetic variation from exotic germplasm or Chinese land races into US germplasm (e.g. Li *et al.* 2008), achieving a quantum leap in soybean yields and yield potential will almost certainly require biotechnological advances that enable improvement of multiple traits. We outlined a number of potential targets, including improved photosynthetic and respiratory efficiency, increased sink strength potential and allocation of C and N to developing pods, synchronized floral initiation to promote greater pod survival and optimized soybean–*Rhizobia* compatibility (Fig. 4). Many of these targets have not been tested in soybean or other crops under field conditions, and there is a critical need to bridge the gap between bench science and yield gains in the field. All of the strategies for improving soybean yields are hypothesized to boost production in today's environment, but it is also important to consider that the environment in 2030 will be different from that of today. The future growing conditions for soybean will likely be warmer, precipitation is expected to be more variable, the concentrations of CO₂ and ozone in the atmosphere will be higher, and pests, pathogens and weed competition will likely be altered (Easterling *et al.* 2007). Therefore, improving stress tolerance in soybean will be another critical feature of maintaining and improving yields under a more variable and rapidly changing environment (Tubiello, Soussana & Howden 2007; Ainsworth, Rogers & Leakey 2008; Mittler & Blumwald 2010).

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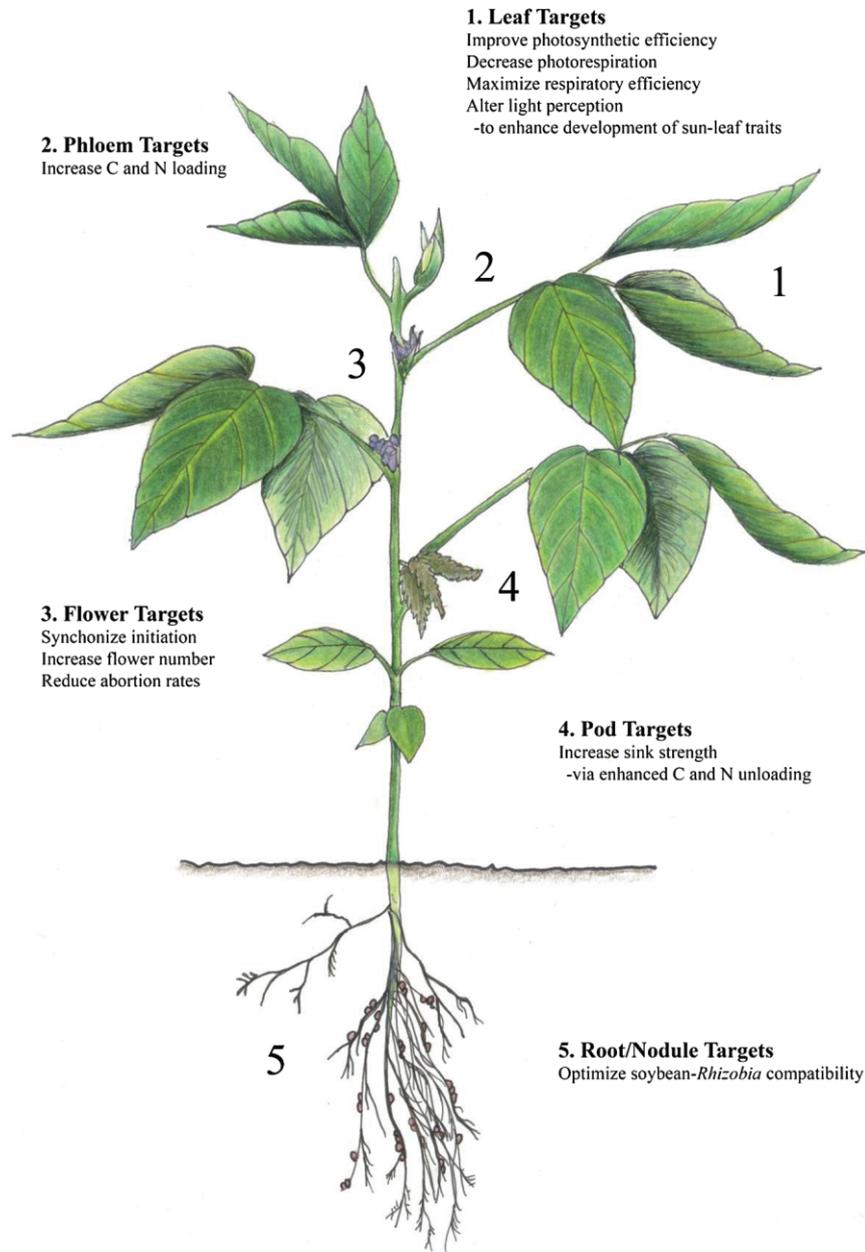


Figure 4. Potential targets for yield enhancement in soybean. A general strategy to improve yield potential is to engineer soybean with (1) greater photosynthetic efficiency, including optimizing ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco), increasing ribulose-1,5-bisphosphate (RuBP) regeneration via SBPase, reducing photoinhibition and altering the photosynthetic electron transport chain. Increasing the sink-strength of developing soybean pods is another putative approach to enhance yield potential. This consists of (2) increasing photoassimilate loading to the phloem in source leaves and (4) unloading in the seeds (3) controlling transition to flowering and increasing flower survival, as well as (5) utilizing more efficient nitrogen fixing *Rhizobia*. Finally, it may be possible to alter flux through the TCA cycle in order to (1) push photoassimilate into phloem and (4) pull photoassimilate into embryos. (Soybean drawing by Elizabeth Yendrek).

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