Field trials to phenotype transgenic tobacco plants with improved RuBP regeneration

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Summary

Evidence has accumulated indicating that improving photosynthetic capacity can lead to increase in yield. The aims of this project were firstly to produce and identify homozygous transgenic tobacco plants and then to test these plants in field conditions for improved photosynthesis and yield. Transgenic *Nicotiana tabacum* plants were produced using gene constructs designed to either overexpress the native H-protein of the glycine cleavage system or express the foreign genes for the cyanobacterial bifunctional FBPase/SBPase and algal cytochrome c_6 proteins. The bifunctional FBPase/SBPase protein catalyses two steps in the regenerative phase of the Calvin-Benson-Bassham cycle. The H-protein upregulates the complex that facilitates the conversion of glycine to serine in the photorespiratory pathway, while the cytochrome c_6 protein is an electron transport protein that assists the flow of electrons from the cytochrome b_6f complex to photosystem I. These plants were tested in field conditions as single, double (bifunctional FBPase/SBPase + cytochrome c_6) and triple (bifunctional FBPase/SBPase + cytochrome c_6 + H-protein) gene manipulations. To determine if the transgenic plants had an increase in productivity over the wildtype and whether combining manipulations could lead to an additive/synergistic increase in productivity. A preliminary trial was held, that targeted single manipulations, which demonstrated significant increases in biomass over the control plants of between 25 to 40%. Due to these results, fully replicated trials were held 2017 and 2018 seasons, where single, the double and triple manipulations were trialled in unison. The 2017 field trials provided maximum increases in biomass of 8% for the single manipulations whereas this was around 18% for the double and triple manipulation. This data provided the strongest evidence that multiple manipulations have a increased effect on growth and productivity than single manipulations. The next stage is to test these manipulations in crop plants and in a range of environmental conditions.

Abbreviations

1,3-BPGA	1,3-Bisphosphoglyceric acid
2-PG	2-phosphoglycolate
3-PGA	3-phosphoglycera
A	Assimilation
ADP	adenosine diphosphate
ATP	Adenosine triphosphate
BAP	6-Benzylaminopurine
C3 cycle	The Calvin-Benson-Bassham Cycle
C_6	Cytochrome c_6
CCM	carbon concentration mechanism
cDNA	Complementary DNA
Ci	internal carbon concentrations
C_i	internal carbon dioxide concentrations
CN	control plants
Cyt b_6f	cytochrome b_6f
D	Double manipulation
DAS	Days after sowing

DHAP	dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
F-1,6-BP	Fructose 1,6-bisphosphate
F-6-P	fructose-6-Phosphate
FACE	Free-air CO_2 enrichment
FBPase	fructose 1,6-bisphosphatase
FBPA	Fructose-bisphosphate aldolase
G3P	glyceraldehyde-3-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCS	glycine cleavage system
GDC	glycine decarboxylase
gs	Stomatal conductance
IAA	1-Naphthaleneacetic acid
ictB	inorganic carbon transporter B
IRGA	infrared gas analyser

iWUE	Intrinsic water use efficiency
LB	Lysogeny broth
MEP	2-C-methyl-d-erythritol 4-phosphate
MS	Murashige and Skoog medium
NAA	3-Indoleacetic acid
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NPQ	Non-photochemical quenching
PC	Plastocyanin
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
PGP	2-phosphoglycolate phosphatase
P_i	Phosphate
PQ	Plastoquinone
PsbS	Photosystem II subunit S
PSI	Photosystem 1
PSII	Photosystem 2
q	Light

qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RuBisCO	ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose 1,5-bisphosphate
S-1, 7-BP	sedoheptulose-1, 7 bisphosphate
S-7-P	sedoheptulose-7-phosphate
S_B	Bifunctional FBPase/FBPas
SBPase	Sedoheptulose-1, 7-bisphosphatase
T	Triple manipulation
T-DNA	Transfer DNA
TK	Transketolas
VPD	vapour pressure deficit

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Chapter 1 – Introduction

1.1 Introduction into the need for crop improvement

The predicted increase in food production needed to feed the world population in year 2050 is expected to be doubled of what was produced in 2010 (Tilman et al., 2011). This is due to several factors. First and foremost, as the population increases, the amount of food required will also increase (Foley et al., 2011, Tilman et al., 2009). Population growth is a multifaceted problem, not only is there a direct increase to the population, but around 95% of the population growth will be in the less developed world. Each year, an increase of around 22 million people are being absorbed into the least developed countries, where food insecurity is the highest (FAO, 2014).

Another factor that calls for an increase in food production is the shift in global diets. The trend for a more meat-based diet vastly increases the amount of livestock food that needs to be produced, as opposed to a plant-based diet. This combined with a global trend of consuming more calories will lead to the large increase in global food demands (Long et al., 2015, Ort et al., 2015).

For now, agricultural yields have managed to keep up with the increasing food demand. The number of people living in food insecurity decreased by approximately 200 million people over a 10-year period, between 1992 and 2002 (FAO, 2014). Given the projected increase in food demand, this means that further increases to yield is needed in the coming years.

Recent increases in food production are mainly attributed to better agricultural practises, better yielding crops and the clearing of land for agricultural purposes. The ‘Green Revolution’ were a large shift in agriculture productivity, which went over a twenty-year period starting in the early 1950’s. As such, a large increase in productivity per unit area was achieved by selecting plants that exhibited a dwarfed phenotype. These did not grow

as tall or large as other plants, but instead the resources were used for the production of grain (Hedden, 2003). Higher yielding plants, combined with smart applications of pesticides and fertilisers, produced a sustained increase in yield. However, these increases in yield have plateaued over the past couple of decades (Zhu et al., 2010, Ort et al., 2015, Ray et al., 2013).

To optimise the food production over the coming years, feasible opportunities must be identified and considered based on return and plausible consequences. One key consideration is the use of land and potential geographical constraints.

One option is to clear more of land to free more space of where food could be produced. However, this comes at a significant cost to the natural environment. The direct impact is the reduction of biodiversity and the loss of habitats that are fundamental for unique species of animals and plants. The indirect impacts of repurposing natural land, including soil loss and increased land erosion, as well as the environmental impact due to increased CO_2 emissions. Soil erosion can be critical for the crop production and could be caused by deforestation or cutting down trees. The root system acts as a mesh to keep the soil in place, but when the trees are removed, the root system anchoring the soil will die. An estimation of soil loss (in 1980) in the Indonesian island of java fount that 770 million tons of topsoil was being lost each year. The loss of this nutrient rich soil equated to an estimated reduction of 1.5 million tons of rice (Magrath, 1989).

With clearing of land posing multiple challenges, the other option would be increasing yield in a more limited space. By engineering or breeding higher yielding plants, it could be possible to produce more food in a smaller area, while also using less fertiliser and pesticide. The agricultural industry is recognised as one of the leading causes of greenhouse gas emissions. Therefore, converting natural land into agricultural land has

a detrimental impact, not only in terms of deforestation, but also increased agricultural emissions (Canadell et al., 2007). However, other factors that produce greenhouse gasses from plant-based agriculture include, CH_4 and N_2O from the burning of residual agricultural by-products, as well as CH_4 from rice cultivation and N_2O released from the fertilisers that are applied to soil (Burney et al., 2010). This means that greenhouse gas emissions, per calories produced, could be reduced by a more efficient use of land for crop production as this would require less fertiliser and pesticide.

Despite the improvements to agricultural practices over the years, traditional breeding has not select for better photosynthetic efficiency, only an increase in yield. The higher yields can be attributed to better land management, and more productive crops. Selective breeding has also produced higher yielding and quicker growing crops that produced more grain per unit area. Interestingly, both CO_2 enrichment studies and gene manipulation studies, have demonstrated that there is scope to improve the efficiency of photosynthesis and how this could contribute to the production of higher yielding crops (Ainsworth and Ort, 2010, Long et al., 2006, Raines, 2006, Simkin et al., 2017a, Simkin et al., 2019, Zhu et al., 2010) .

Photosynthesis is the processes that utilises light energy and inorganic carbon to produce the organic compounds needed for the function and development of plants and other photosynthetic organisms. The ability of a plant to efficiently capture and convert light energy into metabolites is an important determinant that is reflected in the yield of the organism. A crop can only attain its maximum yield when subjected to perfect growing conditions, meaning the plant will have to be grown in the absence of all stress, both biotic and abiotic, and with water and nutrient supplies being non-limiting. The maximum yield attainable by a plant is termed the yield potential (Zhu et al., 2008). Yield potential has

four factors that contribute to the ability of a plant to reach this maximum yield. The abiotic factor that is (i) the light energy available, and three biotic factors which are (ii) the efficiency of light capture, (iii) the conversion of light into biomass and (iv) the proportion of biomass that is partitioned into the desired yield of the crop (Zhu et al., 2008). Traditional plant breeding has greatly improved portioning of biomass into yield, via dwarfing of crops, and the light interception of plant, hence these factors only have a limited impact on yield. Converting the intercepted light into biomass has not been maximised with traditional breeding techniques, and this has been demonstrated with several experiments. Growing plants in CO_2 enrichment experiments provide consistent evidence that increasing carbon assimilation provides increasing to yield (Kohler et al., 2017a, Rosenthal et al., 2011). This is supported by genetic manipulations to photosynthesis. Increasing the rate of photosynthesis, from a molecular approach, has provided evidence of enhancing the productivity of plants (Simkin et al., 2017a, Driever et al., 2017).

1.2 The processes involved in photosynthesis

Photosynthesis can be divided into two main processes that are often referred to as the 'light independent reaction' (electron transport chain) and the 'light reaction' (the Calvin-Benson-Bassham cycle). The light reaction suggests the need of light input for the process to occur, whereas the dark reaction is powered by the products of the light reaction.

1.2.1 Electron transport chain (ETC)

The role of the ETC within photosynthesis is to produce chemically stored energy, that the plant will use in the Calvin-Benson-Bassham Cycle (C3 cycle) (amongst other processes). Sunlight is absorbed by chlorophyll in the two different photosystems (PSII and PSI) that are located in the chloroplast thylakoid membranes. It is the sunlight that

power the ETC and provides the energy to produce ATP and NADPH. The pigment that absorbs light is chlorophyll, and the chlorophyll is located in two distinct reaction centres, photosystem II (PSII) and photosystem I (PSI). The energy absorbed by the chlorophyll is directed to reaction centres which excite an electron. Electrons in the higher energy states can leave the photosystem and reduce a neighbouring molecule. As an electron leave PSII, it needs to be replaced and this is completed by the fission of water. When the oxygen-evolving complex splits water, an electron and proton are released as well as O_2. The electron returns back into PSII. Throughout the electron transport chain, a surplus of protons builds up in the lumen, as previously stated, the fission of H_2O produces H^+ ions and others are recovered from the stroma as the electron passes through the plastoquinone. The light that PSI captures re-excites the electron. This electron is transferred to the ferredoxin (located in the stroma) and then ferredoxin-NADP reductase, this enzyme catalysis the formation of NADPH from $NADP^+$ and H^+. The excess of H^+ ions in the lumen create an electrochemical potential gradient between the lumen and the stroma. Because of the gradient, H^+ ions diffuse through the organelle, ATP synthase, and this diffusion of protons powers the organelle to produces ATP from ADP and P_i.

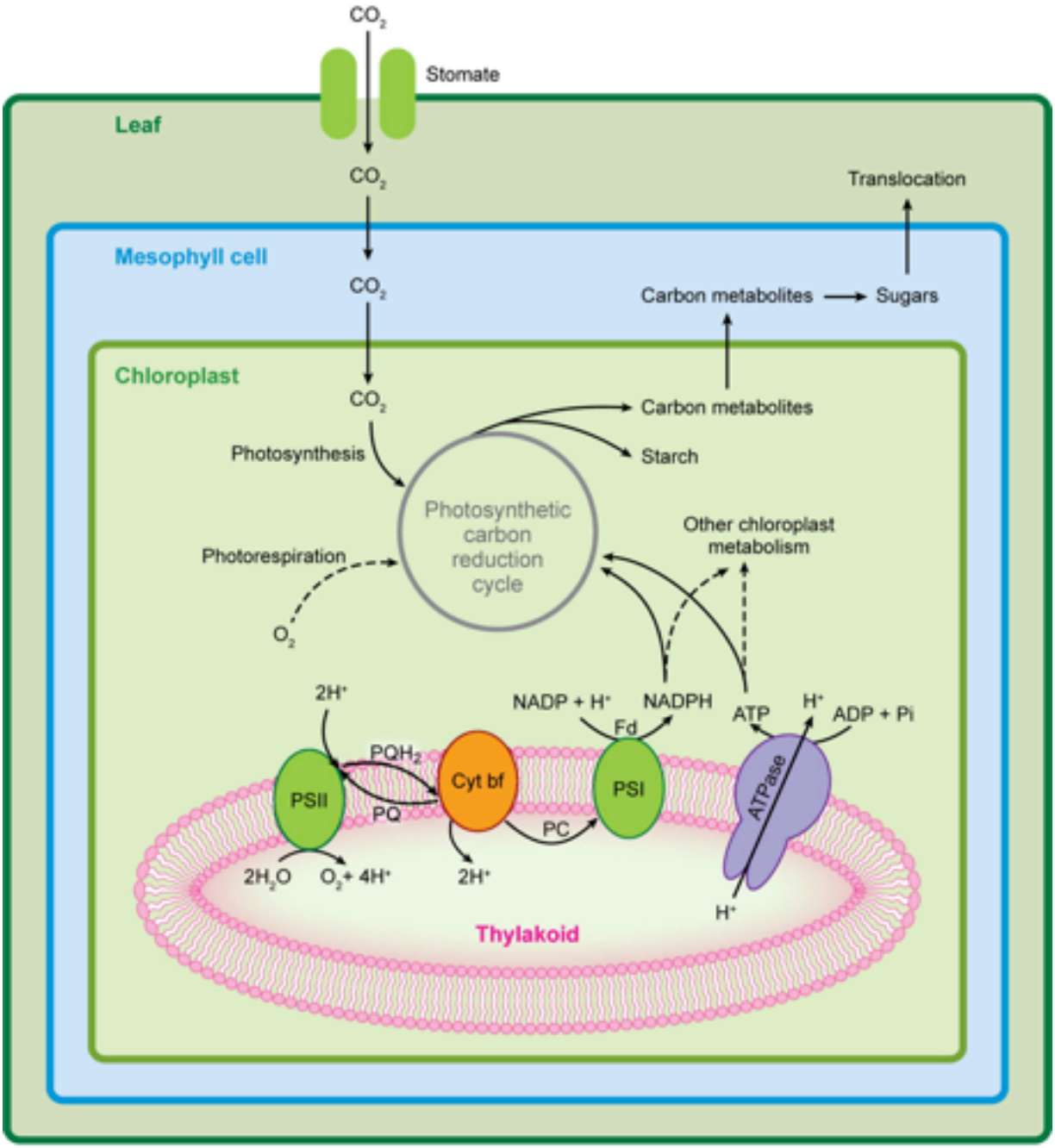

CO_2
Stomate
Leaf
CO_2
Translocation
Mesophyll cell
CO_2
Carbon metabolites
Sugars
Chloroplast
CO_2
Carbon metabolites
Photosynthesis
Starch
Photosynthetic carbon reduction cycle
Other chloroplast metabolism
Photorespiration
O_2
$2H^+$
NADP + H^+
NADPH
Fd
ATP
H^+
ADP + Pi
PQH_2
PSII
Cyt bf
PSI
ATPase
PQ
PC
$2H_2O$
O_2 + $4H^+$
$2H^+$
H^+
Thylakoid

Figure 1.1 The linear electron transport chain. Chlorophyll located in the two different photosystems (PSII and PSI) absorb light which increases the electronegativity of an electron in the PS, electrons in the higher energy states can leave the chlorophyll and reduce a neighbouring molecule. PSII absorbs light with a wavelength of 680 nm or below, this energy is transferred to P680, which excites an electron, the higher energy electron is it transferred to the plastoquinone (PQ) through a series of carriers. The fission of water by the oxygen-evolving complex replenishes the electron back into PSII while producing H+. The PQ diffuses along the thylakoid membrane from PSII to the cytochrome b6f (Cyt b6f) complex, where it donates the electron. This reaction in the PQ recovers a H+ from the chloroplast stroma and releases it into the thylakoid lumen. The electron is transferred through the Cyt b6f complex and into the plastocyanin (PC), which donates the electron to PSI. This process, of shuttling the electron from PSII to the PSI, happens down an electrochemical gradient. PSI absorbs light at 700nm, this energy is transferred to P700 where it re-excites the electron. This electron is transferred to the ferredoxin (located in the stroma) and then ferredoxin-NADP reductase, this enzyme catalysis the formation of NADPH from NADP and H+. A surplus of H+ ions in the thylakoid lumen is created through the fission of H2O, the PQ reacting with H+ in the stroma and releasing it in the lumen and the synthesis of NADPH using the H+ in the stoma. The created electrochemical gradient powers the diffusion of protons through ATP synthase which creates ATP. Image taken from Baker (2008)

1.2.2 The Calvin-Benson-Bassham Cycle (C3 Cycle)

The C3 cycle is a series of 13 reactions that utilise atmospheric CO_2 and convert this into organic carbon compounds that are essential to the metabolism of plants. The C3 cycle occurs in the stroma of the chloroplast and can be split into three main stages.

Stage 1, Carboxylation:

Carboxylation utilises ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) to catalyses the reaction between atmospheric CO_2 and RuBP, to create two molecules of 3-phosphoglycerate (3-PGA). The name of the C3 cycle is derived from this reaction as 3-PGA is a three-carbon molecule.

Stage 2, Reduction:

3-PGA is reduced to 1,3-Bisphosphoglyceric acid (1,3-BPGA), which requires ATP. The reducing power of NADPH is used to produce glyceraldehyde-3-phosphate (G3P) from 1,3-BGPA. These two reactions are catalysed by Phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) respectively. For every CO_2 molecule introduced into the C3 cycle, 2 molecules of G3P are produced. G3P can be exported from the C3 cycle for the 2-C-methyl-d-erythritol 4-phosphate (MEP) pathway, for the production of many compounds including isoprenoids (Phillips et al., 2008).

Stage 3, Regeneration:

The third part of the cycle is series of reactions in secondary pathways that remake the acceptor molecule, RuBP. There are a number of pathways that utilise carbon from the C3 cycle during the regeneration stage, including starch/sucrose syntheses, the products of the shikimate pathway and the thiamine nucleotide reaction.

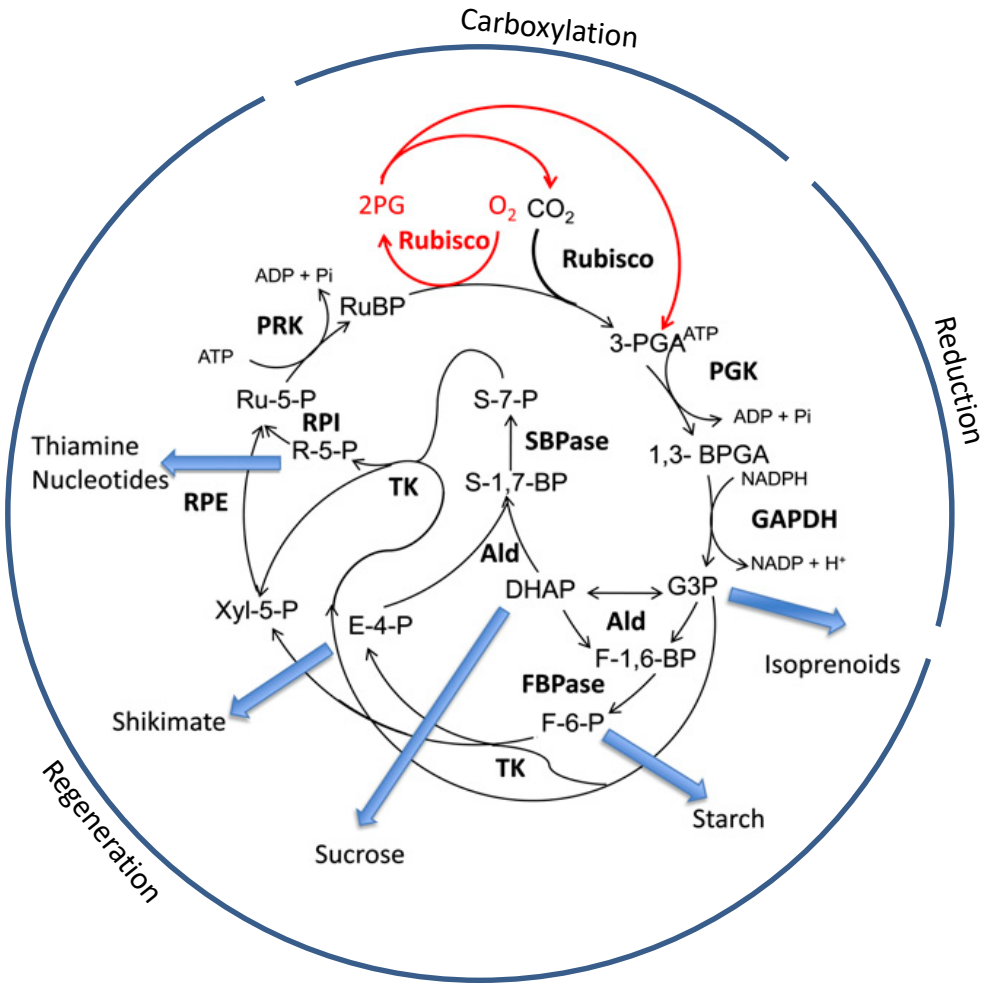


Figure 1.2 The Calvin-Benson-Basshan cycle (modified from Raines, 2011). The carboxylation phase of the C3 cycle uses RuBisCO as the catalyst to fix a CO_2 molecule to RuBP, which forms 3-phosphoglyceric acid (3-PGA). The reduction phase follows, this convers 3-PGA to glyceraldehyde-3-phosphate (G3P). The regeneration phase uses aldolase (ALD) to catalyse the reaction that forms either dihydroxyacetone phosphate (DHAP) or Fructose 1,6-bisphosphate (F-1,6-BP). Either FBPase or SBPase catalyse DHAP to produce fructose-6-Phosphate (F-6-P) or sedoheptulose-7-phosphate (S-7-P). These compounds pass through a series of reactions to produce RuBP, completing one cycle.

1.2.3 Photorespiratory pathway

Photosynthetic carbon assimilation directly competes with the photorespiratory pathway, due to the polygamous nature of Ribulose 1,5-bisphosphate (RuBP). RuBP is the acceptor molecule for atmospheric CO_2, which initiates carbon fixation, but is also an acceptor molecule for atmospheric O_2. When O_2 reacts with RuBP it produced compounds toxic to the plant, unless metabolised. This process reduced the efficiency of carbon metabolism as it consumes energy and utilises carbon that would otherwise be used in carbon assimilation.

The Carboxylation reaction of RuBP produces two three carbon molecules (3-PGA) that enter into the C3 cycle. Whereas the oxidative reaction of RuBP generates one molecule of both 3-PGA and 2-phosphoglycolate (2-PG) (Bowes et al., 1971, Ogren and Bowes, 1971, Somerville and Ogren, 1979, Lorimer, 1981). It is necessary for plants to metabolise 2-PG due to this molecule being toxic to the organism when in sufficient concentrations. When 2-PG accumulates, it reduces the functionality of phosphofructokinase and triose phosphate isomerase, and this inhibits the plants ability to reconstitute RuBP (Kelly and Latzko, 1976, Artus et al., 1986, GonzalezMoro et al., 1997). The molecule of 2-PG is dephosphorylated by 2-phosphoglycolate phosphatase (PGP), which produces glycolate. Glycolate is transported into the peroxisome where it is converts to glyoxylate and then to glycine. Glycine is moved into the mitochondria where the glycine cleavage system (GCS) (also known as glycine decarboxylase (GDC)) converts the glycine to serine, which releases CO_2 while producing ammonium. Serine is transported back into the peroxisome and is converted to glycerate through multiple steps. Glycerate is moved into the chloroplast where it is phosphorylated and enters the C3 cycle a 3-PGA.

Photorespiration is a consumer of energy and organic carbon, while diverting RuBP away from the C3 cycle. It is estimated that the energy used to process every completed cycle has the energetic equivalent to 12.25 ATP molecules. The specificity of RuBisCO favours the carboxylation reaction over the oxygenase reaction with a specificity ratio of 0.8:0.2. However, due to the much higher concentration of oxygen in the atmosphere the actual rate of oxygenation to carboxylation is about 0.25:0.75 respectively. But as the carboxylation reaction uses the energy equivalent to around 4.1 ATP molecules, the total energy expenditure is around 0.5:0.5 for both of the competing reactions (Peterhansel et al., 2010).

The rate of oxidation of RuBP is affected by several factors, and an increase in temperature will decrease RuBisCO specificity, while water stress cause stomata to close which increases photorespiration by decreasing internal carbon concentrations (*Ci*) (Ku et al., 1977, Jordan and Ogren, 1984). These conditions have a high energetic cost and plants growing in these conditions can use up to 50% of the ATP in photorespiration (Walker et al., 2016).

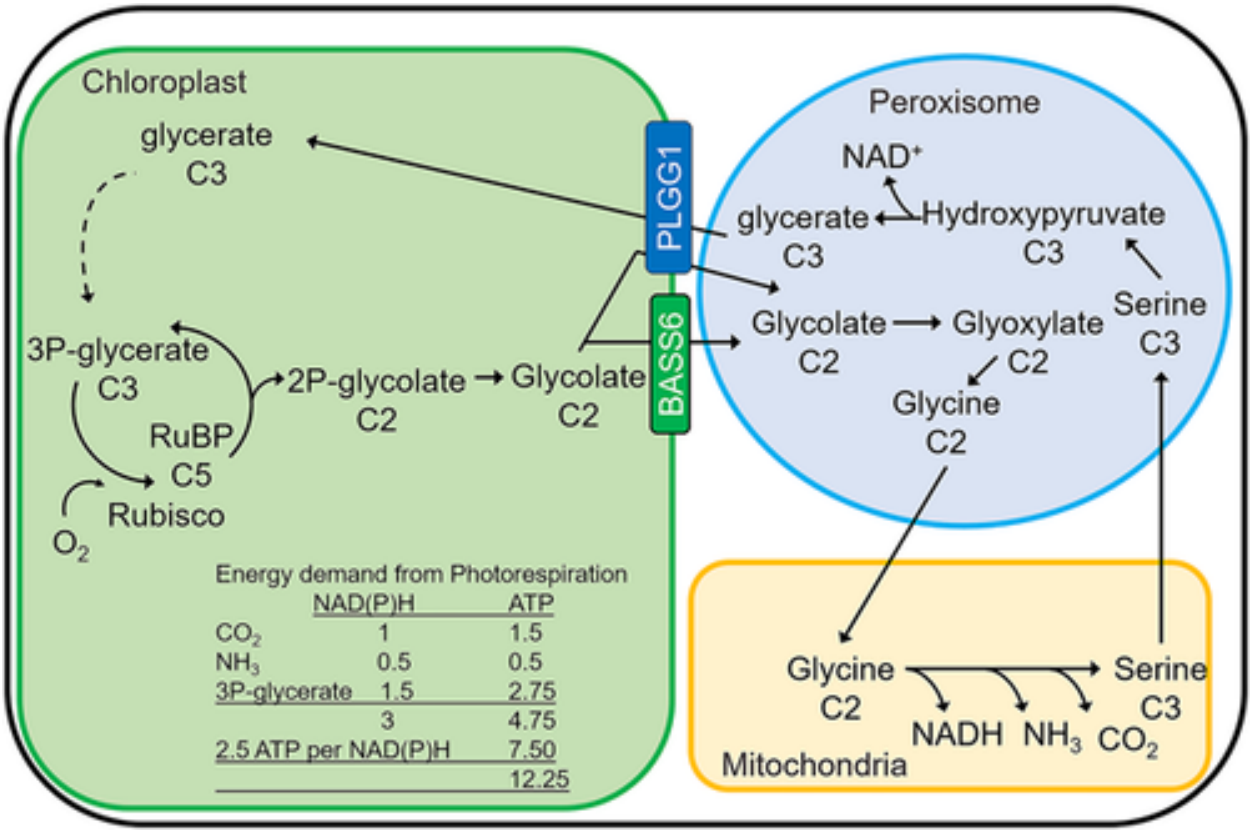


Figure 1.3. The process involved in photorespiration (South et al., 2018). Photorespiration happens in the cytosol (White). The green organelle represents the chloroplast, the white represents the peroxisome and the yellow represents the mitochondria. The oxygenation reaction of RuBP creates 2P-glycolate (2-PG) , for every 2 molecules of 2-PG created, one returns to the C3 cycle as 3PG and one is lost as CO_2. The image originated from South et al. (2019) with the energy demand included from Perterhansel et al. (2010)

1.3 Evidence that manipulation of RuBP regeneration can improve yields

A large proportion of the limitation to the C3 cycle comes from the inefficiency of RuBisCO, but the limitation imposed by RuBisCOs is not all encompassing. A review by Raines (2011) highlighted several enzymes that had control over the flux of carbon through the C3 cycle. As well as RuBisCO, the enzymes noted for potentially having the most control over the rate of carbon flux through the C3 cycle were sedoheptulose-1,7-bisphosphatase (SBPase), Transketolase (TK) and Fructose-bisphosphate aldolase (FBPA). Experiments downregulating the expression of these enzymes, as well as others including fructose 1,6-bisphosphatase (FBPase), provided evidence of the potential that enzymes in the C3 cycle other than RuBisCO could control carbon flux under certain environmental conditions. These downregulation experiments produced plants that had a reduced growth phenotype (Harrison et al., 1997, Lawson et al., 2006, Kossmann et al., 1992, Haake et al., 1998). However, this does not necessarily provide direct evidence that an increase in the level of these enzymes would increase photosynthesis and produce higher yielding plants.

Early work in the overexpression of enzymes in the C3 cycle, to improve photosynthesis, focused on upregulating the activity of individual proteins. Some of the most successful early experiments overexpressed SBPase. This enzyme is a nuclear encoded gene that catalysis the conversion of sedoheptulose-1, 7 bisphosphate (S-1, 7-BP) to sedoheptulose-7-phosphate (S-7-P). The effects of upregulating this gene has been demonstrated in a variety of plants. Lefebvre et al. (2005) produced *Nicotiana tabacum* plants with an increase in SBPase activity, and this change in activity had a significant increase in carbon assimilation, which resulted in an increase of up to 30% in biomass. The demonstrated improvements in productivity from Lefebvre et al. (2005) was shown to be conserved when the plants were grown in high and low light experiments, several

years after the initial trials (Simkin et al., 2015). More recently the over expression of SBPase in *Arabidopsis thaliana* provided a similar phenotype to that observed in tobacco, an increased in photosynthesis and an increase in biomass (Simkin et al., 2017a). What this has demonstrated is that the improvement RuBP regeneration, which leads to an increase in biomass, is not species specific.

Improving the productivity of plants in model species provides good evidence of the potential that the overexpression of the gene could have on the yield of plants, but this impact is measured in terms of biomass increases in the leaves and stem of the plant, and this is may not be relevant to crop species. When SBPase is overexpressed in tomato it led to similar improvements to productivity that were observed in tobacco (Ding et al 2016) but extended this work by demonstrating an increase in the yield of fruit. The overexpression of SBPase was carried out in wheat by Driever et al (2017) and plants were produced overexpressing SBPase and growth analysis was undertaken in two different growing conditions. The first was a regime that encouraged tillering, by growing plants in low densities, and the other was the opposite to this, growing plants in a high-density environment to discourage tillering. The yield phenotype differed in the growth regimes, with plants that grew in high density had an increase in seeds per ear, over the controls. Plants that grew in low density did not show an increase in seeds per ear but produced more ears when compared to the controls. Under both growing condition overexpressing SBPase provides benefits to the yield of wheat of up to ~40%. This result demonstrates that increase in biomass in model species has the possibility of increasing yield in crop species. The overexpression of SBPase has provided evidence that elevating the rate of RuBP regeneration can improve productivity of crops, both monocots (Driever et al., 2017) and dicots (Ding et al., 2016). However, if one bottle neck to

productivity is removed, could removing another have an additive affect to the rate of photosynthesis and growth?

Analysis of multiple gene manipulation carried out in *N. tabacum* demonstrated an additive effect to productivity of expressing multiple genes involved in photosynthesis. Plants were made expressing/overexpressing SBPase, FBP aldolase and ictB. IctB is protein that is highly conserved in cyanobacteria (Bonfil et al., 1998), and although it was originally thought to be an inorganic carbon transporter, its function is still unknown (Price et al., 2011). Expression vectors were created as single gene constructs and together in double or triple gene transgenic plants. Every transgenic line had an increase in biomass over the control plants, but interestingly when the genes were expressed in unison the plant had a greater biomass. So, plants expressing SBPase alone had an increase in productivity over the controls but when compared to plants expressing two or three of the genes, SBPase plants were significantly smaller.

1.4 Transgenic approaches to minimise productivity loss due to photorespiration

Carboxylation of RuBP is catalysed by RuBisCO, but RuBisCO also catalyses the competing oxidative reaction of RuBP. RuBP has a high affinity to CO_2, but due to concentrations of CO_2 and O_2 in the environment, the oxidation reaction occurs in around 25% of the time. The oxidative reaction has a low turnover rate, so in high O_2 conditions the competing oxidative reaction can completely inhibit the carboxylation reaction from being catalysed (Hartman and Harpel, 1994). It is estimated that there is up to a 50% loss in carbon fixation due to the oxidative reaction of RuBP (Zelitch, 1973).

Attempts to reduce photorespiration, both chemically and genetically, have demonstrated how this pathway is an important regulator of the C3 cycle, in atmospheric conditions. So, impairing the photorespiratory pathway reduces the rate of carbon fixation (Servaites,

1977, Wingler et al., 1997). Timm et al. (2012) proposed the converse to this, that expediting the process could increase the rate of carbon fixation. Fundamental to photorespiration, the glycine cleavage system (GCS) is a set of four proteins, three being enzymes (subunits: P, T and L), and one messenger/communicating protein between the three enzymes, the H-protein subunit. This complex of proteins is essential in the conversion of glycine to serine. When overexpressing the H-protein in *vitro* there is an upregulation of other components of the complex (Hasse et al., 2009). Studies overexpressing just the H-protein in, *Arabidopsis thaliana,* lead to an increased growth rate in terms of rosette area, leaf number and leaf size. This culminated in dry biomass increases up to 1/3 for the best performing lines (Simkin et al., 2017a).

As photorespiration is used to metabolise the toxic compound produced from the oxygenase reaction of RuBP and O_2, it would be possible to use a RuBisCO with a higher affinity to CO_2 to mitigate against the oxidative reaction. A common trade of in RuBisCO has been observed in that isoforms with a higher specificity to CO_2 display lower kinetic performance. This means any expected increase in productivity is negated by lower kinetics, or any increase in RuBisCO kinetics is negated by a lower specificity to CO_2. Not all organisms have demonstrated the trade-off between kinetics and CO_2 specificity, analysis of form I RuBisCOs in diatoms demonstrated little difference in oxidation rates, irrespective of carboxylation speed (Young et al., 2016).

There were difficulties in initial attempts to introduce a foreign RuBisCO into plants. This was due largely to the fact that the small subunit is encoded in the nucleus and the large subunits plastid encoded. When genes encoding for the large subunit were transformed into the nucleus of the plant, there was poor RuBisCO accumulation in the chloroplast (Lin et al. 2018). Advances in technology have led to plastid transformation being

achievable. Unfortunately, selective marker un-linkage would occur providing additional problem with producing transformants, as the selective marker genes would be incorporated into the plastid, and not the RuBisCO genes, leading to false positive transformation events. However, transgenic tobacco was created with a RuBisCO large subunit being expressed in place of the native, however, these plants did not grow well in ambient [CO_2]. The RuBisCO large subunit transformant was subsequently used as a masterline, for retransformed with alternative RuBisCO, transformants could be selected for by their ability to grow under ambient air conditions, with the non-transformed plants unable to grow. This masterline has enabled as large range of rubisco large sub unit transformations to be created (Sharwood, 2016). Having the ability to phenotype foreign RuBisCOs *in vivo* provides information on their phenotype, which has relevance when selecting better RuBisCOs for plant transformation.

Finding a more suitable RuBisCOs is one way to reduce photorespiration, however, organisms that live in areas with low levels of [CO_2] have developed alternative solutions, CO_2 diffuses much slower in water than in air, as such, photosynthetic organisms living in water can sometimes find themselves in an environment with limited available carbon. To overcome the lack of CO_2, organisms have evolved carbon-concentrating mechanisms (CCM's) that concentrate the available carbon around RuBisCO in the chloroplast. By concentrating around the RuBisCO protein, the rate of photorespiration is reduced, making carbon fixation more productive. Two types of CCMs include carboxisomes and pyrenoids, commonly found in bacteria and algae respectively. Although with different structure, both perform the same function, by using carbon transporters to move dissolved inorganic carbon in to the CCM, and releasing it from solution, via carbonic anhydrase, making it available to RuBisCO.

CCMs have potential to be introduced in to higher plants, in an attempt to mitigate the losses of efficiency due to photorespiration. Producing functional carboxisomes has been difficult due to around 12 genes being needed to for its assembly, however functional carboxisomes have been produced in plants, albeit, with a bacterial RuBsiCO (Long et al., 2018). However, due to the plants having bacterial RuBisCO, they need to be grown in elevated CO_2 conditions. Although in its infancy, this work demonstrates that CCMs can be introduced into plants.

A separate approach to increase photosynthetic rates and plant biomass, via manipulation of photorespiration, was demonstrated by introducing an alternative pathway for 2-PG metabolism. Plant malate synthase and an algal glycolate dehydrogenase, that were expressed in the chloroplast to metabolise 2-PG, demonstrated an increase biomass of >25%. This pathway was also tested in plants that downregulated the native photorespiratory pathway, which led to an increase in biomass of >40% (South et al., 2019). As this was demonstrated in field conditions, which exhibits additional stress on the plants, it suggests the photorespiratory pathway does not play to pivotal role in the health of the plant, apart from metabolising the toxic compound produced for the oxidative reaction of RuBP.

1.5 Alternative approaches to improving carbon metabolism

Under high levels of light or CO_2, it is the regeneration of RuBP and not the carboxylation of RuBP that limits the flux of carbon though the C3 cycle (Farquhar et al., 1980). Cytochrome c_6 (C_6) functions as a component to transfer electrons between the cytochrome b_6f complex and the reaction centre in photosystem I in some cyanobacteria and algae (Fig. 6). It is widely accepted that the plant-based cytochrome c_6 was evolutionarily discarded (Sigfridsson et al., 1998, De la Rosa et al., 2002). Yet, genomic

analysis has found a similar protein in higher plants (De la Rosa et al., 2006). However, the view that this acts as an electron transporter between cytochrome *b_6f* and PSI has been discarded (Chida et al., 2006), meaning it potentially may be reintroduced to improve the rate of the electron transport chain.

Cytochrome *c_6* has been expressed in *Arabidopsis thaliana*, from a cyanobacteria (*Porphyra yezoensis*) and "thus going back in the evolutionary history of plants" (Chida et al., 2007). This entails that a higher plant had both *C_6* and, the native, copper protein plastocyanin (PC), another electron transporter. Introducing *C_6* demonstrated the opportunity to increase photosynthesis through the turbocharging the ETC (Chida et al., 2007).

It is possible to manipulate the ETC with native genes, as well as introduced genes. The downregulation of the rieske FeS protein, a part of the cytochrome *b_6f* complex, saw correlated decreases in photosynthesis (Price et al., 1995). Interestingly, when this protein is upregulated, the other components of the complex have a higher expression level. The increase in cytochrome *b_6f* complex leads to an increased rate of the electron transport chain, which more importantly significantly increases the biomass and seed yield of the Arabidopsis.

1.6 Increasing plant productivity in field conditions

When looking at experiments aimed at improving photosynthetic efficiency, the study has usually been completed in glasshouse conditions. Due to several factors, including seasonality, cost, facility availability and the amount of work involved in field trails, is was less commonly completed in the past. But due to the importance of the data they provide, the more recent trend has been to complete field trials, in conjunction with glasshouse experiments, to demonstrate a phenotype.

Although field trials are more difficult to conduct, over glasshouse trials, there have been some significant successes as a cause of these studies. Some of the earliest demonstrations came from Free-air CO_2 enrichment (FACE) experiments. FACE experiments are important as running studies in elevated CO_2 provides information on how plants will respond to the future predicted rise in global CO_2 levels. Meta-analysis of FACE experiments from a range of species and habitats demonstrate that in elevated CO_2, plants exhibits increased photosynthetic carbon assimilation, increased quantum yield and a decrease in stomatal conductance (Long et al., 2004). Overall, FACE experiments provide data that demonstrates how an increase in photosynthesis can increase carbon assimilation.

Soybean expressing a bifunctional gene, from a cyanobacteria, that acts with the specificity of both FBPase and SBPase was grown in a range of conditions in FACE field trails. The data demonstrated an increase of yield by manipulating the C3 cycle over three years of trials, using plants grown in both elevated CO_2 and elevated temperatures. This study verified the importance that transgenic plants may hold for a changing climate, as plants expressing this gene could maintain yield levels in an environment that is detrimental to productivity.

Outside of manipulating the C3 cycle studies, using *N.tabacum* have demonstrated a direct link between improving photosynthesis and the positive affect this has on yield. Kromdijk et al. (2016) introduced genes that reduced the recovery time involved with photoinhibition. By increasing non-photo chemical quenching relaxation, it was possible to increase photosynthetic carbon assimilation, which in turn led to plants having a yield improvement of 15% over wildtype plants.

Manipulating photosynthesis to improve the productivity and yield of plants grown in the field has been demonstrated by completely redesigning the photorespiratory pathway. The oxidative reaction of RuBP produced 2-PG, which is inherently toxic to plants. The photorespiratory pathway metabolises this compound and releases around 75% or the carbon back into the C3 pathway. It is evident, however, that plants with alternative pathways for metabolising 2-PG are more efficient in field conditions. Instead of the energy intense set of reactions that comprises the photorespiratory pathway, 2-PG is metabolised by the introduced genes in the chloroplast. When the native mechanism is inhibited, plants grew up to 40% larger than plants without the introduced pathway (South et al., 2019).

Past field trials have demonstrated that it is still possible to improve productivity via manipulation of photosynthesis, even in the variable, stressful and unpredictable conditions that plants are subjected to in the natural environment (Rosenthal et al., 2011, Kromdijk et al., 2016, Kohler et al., 2017b, South et al., 2019).

1.7 Aims of the project and the targets for genetic manipulation

Previous studies have investigated how targeting genes for introduction or overexpression within different pathways relating to photosynthesis affect their physiology. These studies have demonstrated improved rates of carbon fixation, which has led to increases in productivity, growth, plant biomass or yield (Lefebvre et al., 2005, Chida et al., 2007, Rosenthal et al., 2011, Timm et al., 2012). Glasshouse trials have validated this with a variety of transgenic approaches and by using a range of species (Driever et al., 2017, Simkin et al., 2017a, Simkin et al., 2015). Genes specifically targeting the C3 cycle, the ETC and photorespiration have repeatedly been shown to increase biomass in glasshouse experiments (Simkin et al., 2017a, Simkin et al., 2015,

Simkin et al., 2017b,). When multiple genes are manipulated in unison the plants exhibit an additive effect to final harvest biomass. Although field trails have demonstrated manipulations to photosynthesis can improve the yields of plants, manipulations designed to improve RuBP regeneration have not yet been consistently successful in open air conditions. Therefore, the aim of this project is to produce and phenotype transgenic plants that are expressing a limited number of introduced genes involved in RuBP regeneration, electron transport and photorespiration. The genes that will be used for this project are SBPase, cytochrome c_6 and the H-protein of the GCS. The rationale behind this is that by targeting these different processes, the effect of increasing all three genes will be additive or even synergistic and thereby provide a greater effect on yield.

The aims of this project were achieved through the following objectives:

1. Transgenic *N.tabacum* plants were produced using *Agrobacterium tumefaciens*. The transgenic plants were transformed with single gene constructs and phenotyped on a molecular and physiological level. Individual lines that exhibit the desired phenotype will be crossed to produce plants that have a double manipulation (C_6 and S_B) and plants with a triple manipulation (C_6, S_B and H-protein).

2. Transgenic plants with single gene manipulations (C_6, S_B and H-protein) were phenotyped under field conditions to determine the impact each individual manipulation has on the photosynthetic capacity and biomass of the plant.

3. Plants expressing the single manipulations will be repeated to determine the robustness of the manipulation over several seasons.

4. The double (C_6 and S_B) and triple (C_6, S_B and H-protein) manipulations were grown alongside the plants expressing the single gens. This was to determine whether there is

an additive or accumulative effect of expressing several proteins involved in carbon metabolism.

Chapter 2 – Materials and methods

Transgenic Plant Preparation and tobacco transformations

Tobacco (Nicotiana tabacum cv. Petit Havana) was used in all experiments.

2.1 Generating constructs

The constructs used in these experiments were designed and assembled by Patricia E. López-Calcagno, using Golden Gate cloning (Engler et al., 2014), and a description of the assembly is stated in (Lopez-Calcagno et al., Unpublished, Lopez-Calcagno et al., 2019). A range of promoters were used (p35S, cauliflower mosaic virus 35S promoter. ST-LS1, Solanum tuberosum ST-LS1 promoter. pNos, nopaline synthase promoter. pFMV, figwart mosaic virus promoter), in combination with the coding sequences for the Arabidopsis thaliana H-protein (AT2G35370), the codon optimised *P. umbilicalis's* cytochrome c_6 (AFC39870) and the cyanobacterial bifunctional fructose-1,6-bisphosphatases/sedoheptulose-1,7-bisphosphatase (FBP/SBPase; *slr2094*). The terminators used were: tNos, nopaline synthase terminator. tHSP, heat shock protein 18.1 terminator. t35S, cauliflower mosaic virus 35S terminator. The selection markers used were BAR, Bialaphos resistance gene. HTP, hygromycin phosphotransferase gene.

2.2 Seed sterilisation

2.2.1 Sterilisation with bleach

Seeds were sterilized with a 4 % (v/v) bleach solution and 1 drop of tween 20 in 50 ml of solution. The seeds were agitated using a rotating mixer. The seeds were then washed with this solution using a rotating mixer, for 15 minutes before being moved into a class II laminar flow hood and rinsed five times with sterile H_2O.

2.2.2 Sterilisation with ethanol

Seeds were placed in a solution of 95 % ethanol and 0.1 % tween 20 for three minutes and were agitated using a revolving mixer.The seed were then moved into the class II hood and washed a further five times with 75 % ethanol. The seeds were then placed on sterile filter paper and left to air dry before being sown.

2.3 Media composition

2.3.1 Tobacco seed germination

Nicotiana tabcum L. cv Petit havana seeds were germinated in media containing 0.8 % (w/v) agar containing 0.44 % (w/v) Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) with a pH adjusted to 5.9 with NaOH. Sterile magenta pots (Bibby Sterilin Ltd.) were used to germinate four seed per pot.

2.3.2 Rooting media

The rooting media has is the same composition as tobacco seed germination media but with the antibiotic cefotaxime at 400 mg/ml and Hygromycin B at 20 mg/ml.

2.3.3 Transformation media

Transformed leaf disks were grown on plant transformation media containing 0.8 % (w/v) agar, with 0.44 % (w/v) Murashige and Skoog medium (MS) and full strength Gamborg vitamins (Gamborg et al., 1976) with the pH was adjusted to 5.9. Two hormone mixes were used at different stages of the transformation process, NBM and EM. EM media contained 6-Benzylaminopurine (BAP) at 2.5 mg/L and 3-Indoleacetic acid (IAA) at 0.2 mg/L. NBA media used BAP at a concentration of 1 mg/L and 1-Naphthaleneacetic acid (NAA) at 0.1 mg/L.

2.4 Preparation of *Agrobacterium tumefaciens* competent cells

Preparation of the *Agrobacterium tumefaciens* strain LBA 4404 competent cells was completed via the protocol described by Sambrook (2001). A single colony that was grown on a LB (Bertani, 2004) plate containing rifampicin (50 μg/ml) and streptomycin (30 μg/ml) was used to inoculate 10ml of liquid LB, again, containing rifampicin (50 μg/ml) and streptomycin (30 μg/ml). The inoculant was grown in a shaking incubator at 28 °C for 48 hours. The inoculant was chilled and spun down at 3000 g, for 15 minutes at 4 °C. The cells were resuspended in 10 ml ice cold, sterile, ultra-pure water from a Milli-Q water filtration system (Millipore corporation, MA, USA). The resuspended cells were washed by centrifuging them in the same conditions as previous. The washes were completed 4 time after which the cells were resuspended in 200 μl of the ice cold, ultra-pure water, with 10 % glycerol and aliquoted in 40 μl aliquots into sterile 1.5 ml microcentrifuge tubes. The aliquots were either immediately used on stored at -80 °C.

2.4.1 Transformation of agrobacterium strain LBA 4404

2 μl of DNA plasmid mixed into a 40 μl aliquot of competent LBA 4404 cells. This was transferred to a sterile, ice cold, electroporation cuvette. Using an EasyJect Prima electroporator (EQUIBIO), the cells were electroporated at 2500 V. 1ml of ice cold, sterile, liquid LB was added into the cuvette, and then transferred back into the original aliquot tube. The cells were placed into a shaking incubator at 28 °C for two hours. 100 μl of cells was spread onto LB plates containing the necessary antibiotics, the remining cells were spun down and 700 μl of media was removed, and the remaining cells were resuspended and spread onto a plate. The plates were incubated for 48 hours at 28 °C.

Tobacco transformation and regeneration

2.5 Tobacco transformation

Agrobacterium-mediated transformation of *N.tabacum* L. cv Petit havana was carried out on leaf disk in the methodology set out by (Horsch et al., 1989).

A single colony of transformed *A.tumefaciens* was used to inoculate 10ml of LB containing antibiotics. The inoculation was grown over night at 28 ^{o}C in a shacking incubator. 2-4 ml of the culture was used in inoculate a 150 ml of LB containing the required antibiotics. The 150 ml culture was left to grown in a shaking incubator at 28 ^{o}C until an optical density (OD_{600}) of 0.5-1.0 was reached, this usually took 48 hours. The 150 ml was split into three 50 ml sterile centrifuge tubes and centrifuged at 3000 g for 20 minutes. The supernatant was removed, and the cells were resuspended in the equivalent volume of liquid MS media.

Wildtype *N.tabacum* seeds were germinated in sterile pots containing ~40 ml of seed germination media, with four seed per pot. The seedlings were grown in climate control incubator with a photosynthetic photon flux density (PPFD) of 200 μmol m^{-2} s^{-1} for a 16-hour photoperiod at 22 ^{o}C. Six weeks after germination, leaf disks were cut in the range of 1 to 2 cm^2. The leaf disks were placed in the agrobacteria containing liquid MS and left for 30 minutes.

After 30 minutes the leaf disks were moved on plates containing NBM media, without antibiotics, for two days in the dark. The leaf disks were then transferred onto plates containing NBM media with antibiotics. The antibiotics used were cefotaxime at 400 mg/ml and Hygromycin B at 20 mg/ml. Cefotaxime was used to kill the agrobacteria and hygromycin B was used to kill and leaf disks that had not been successfully transformed. After two weeks the leaf disks were moved on to EM media, containing antibiotics, and

left to regenerate while refreshing the media every 7 to 10 days. Once shoots of around 1cm had regenerated form the leaf disks, they moved into rooting media. Four shoots per transformation event were moved into rooting media to increase the change of the regenerated plant surviving. The growth conditions of the incubator were 22 ^{o}C, with a 16 hour photoperiod at an intensity of 200 μmol m^{-2} s^{-1}.

Once a root system had been established, one plant per transformation event was moved into 100 ml pots containing moist compost that had a pH between 5.3 and 6 (Levington F2, Finson, UK). After plants were moved to soil, they were grown in trays acclimatised to the environment with the use of a propagator. The primary transformants were grown in climate control chambers with the environment set to a 16-hour photoperiod and a PPFD of 400 μmol m^{-2} s^{-1}, at 25 ^{o}C. During the 8 hours of dark the temperature was set to 18 ^{o}C. The propagators were removed after three days and the T0 transgenics were kept in the climate control chamber for two weeks before being moved into the glasshouse, where they grew and self-fertilised to produce seed.

2.6 Tobacco Crosses

The crossing of plants was achieved via the transfer of pollen to the stigmas of flowers that had yet to open, through standard protocol. Each cross had between 5 and 10 attempts of fertilisation with different flowers. The petals were cut open and the unmatured stamen were removed before using a mature stamen, from the other plant of interest, to cover the stigma in pollen. A cover of clingfilm was used to protect the opened flower. The F1 generation of plants were screened via PCR for the genes of interest to confirm that both constructs were being transcribed in the cross.

Molecular biology techniques

2.7 Polymerase chain reaction (PCR)

PCR reactions were performed in a 10 µl reaction. The reaction consisted of 6.6 µl H_2O, 0.2 µl dNTPs (10 mM stock), 0.5 µl forward and reverse primers (10 pmol µl-1 stock), 0.2 µl Dreamtaq polymerase and 1 µl of DNA.

2.8 Colony polymerase chain reaction (PCR)

Colony PCR were performed on transformed agrobacterial colonies prior to plant transformation. These PCR's were performed with Dreamtaq (Thermo Scientific). A 15 µl reaction was made consisting of H_2O (2.6 µl), Dreamtaq buffer (1.5 µl), forward and reverser primers (0.75 µl each), dNTPs (0.2 µl at 10 mM stock), Dreamtaq polymerase (0.2 µl) and agrobacterium (DNA) in H_2O (9 µl).

2.9 Agarose gel electrophoresis

DNA was separated using a 1 % agarose gel. TAE buffer was used in both the gel preparation and the running buffer. The final concentration of the TAE was: TRIS 40mM, acetic acid 20mM and EDTA 1mM. Safeview (Nbs biological ltd.) was used for the DNA stain and was imaged under UV light (SYNGENE InGenius3 gel imager).

2.10 RNA/Protein extraction

Two 1 cm^2 leaf disks were ground using the TissueLyser (Qiagen), 1 3 mm ball bearing was added to impact resistant tubes containing the tissue, and ground for 30 seconds at a frequency 30 oscillations 1^{-s}. This process was completed on dry ice, up to placing the samples into the TissueLyser. At no point was the tissue let to defrost.

RNA and Protein was obtained using the nucleospin RNA/Protein kit (Macherey-Nagel, http:// www.mn-net.com/). For full methodology consult their website.

2.11 cDNA synthesis

The RNA obtained from the nucleospin RNA/Protein kit was diluted to 100ng/ μl with RNAse free H_2O. The cDNA synthesis reaction contained: RNA 10 μl, oligo dT's 1μl, dNTP's 2μl, RNAse free H20 2 μl, reverse transcriptase (RevertAid reverse transcriptase, Fermintas) 1 μl and reaction buffer 4 μl. The total volume of the reaction was 20 μl. The heating cycle was 42 ^oC for 60 minutes and 70 ^oC for 10 minutes.

2.12 Quantitative polymerase chain reaction (qPCR)

Primer efficiency was determined by Patricia Lopez-Calcagno. Primer efficiency was determined by diluting the CDNA sample and producing a standard curve as follows: 1, 1:10: 1:100 1:1000 1:10000. Only primers that had an efficiency value between 90 and 110% were used.

The qPCR reactions were performed with the SensiFAST SYBR No-ROX Kit (Bioline Reagents Ltd., London, UK), following manufacturer instructions. The reactions were 15 μl and the mix consisted of: 7.5 μl Bioline sybr mix, 0.75 μl forward and reverse primers (10 pmol μl-1 stock) 6 μl CDNA (2 ng ul-1).

The reaction was run in triplicate with the CFX96 touch system (Bio-Rad) and the cycling conditions for the qPCR were:

- Initial denaturation of 95 ^oC for 2 mins to activate the Taq polymerase
- 35 cycles at 95 ^oC for 5 s for denaturation, annealing at 60 ^oC for 10 s and extension at 72 ^oC for 5 s.

Cq values and primer efficiency were determined using Bio-Rad CFX manager software (v3.1).

2.13 Preparing *Escherichia coli* TOP-10 chemically competent cells

A single colony of the *E.coli* strain, TOP-10, grown on a LB plate was used to inoculate 10 ml of liquid LB. The cells were placed to grow in a shaking incubator over night at 37 ^{o}C. The culture was used to inoculate 100ml of LB which was left to grow, in the same conditions, until it had reached an optical density (OD_{600}) of ~0.4. The culture was centrifuged at 3000 g for 10 minutes and resuspended in 10 ml of ice cold $CaCl_2$ solution (60 mM $CaCl_2$, 15 % glycerol, 10 mM PIPES (Piperazine-1, 4 -bis(2-ethanesufonic acid)) adjusted to pH 7). This step was repeated three time before the cells were incubated for 30 minutes on ice. After the cells have incubated the solution is centrifuged at 300g for 5 minutes at 4 ^{o}C and the supernatant was removed. The cells were resuspended in 4 ml of the $CaCL_2$ solution and then aliquoted in 200 μl into sterile 1.5 ml microcentrifuge tubes. The cells were then either used immediately or were stored at -80 ^{o}C for future use.

2.14 Transformation of E.coli

Chemically competent *E.coli* were transformed using the methodology described in Sambrook (2001). 2 μl of plasmid, at a concentration of 100 g/μl, was added to 200 μl of *E.coli* competent cells and gently mixed before being left on ice for 30 minutes. The cells were heat shocked at 42 ^{o}C for 60 seconds and then put back on ice for a further 2 minutes. 1000 μl of LB (37 ^{o}C) was added and placed in a shacking incubator at 37 ^{o}C for one hour. 100 μl of cells were plated and the remaining solution was spun down. 700 μl of LB were removed and the remaining cells were resuspended and then plated out. The plates were stored in incubation chambers at 37 ^{o}C overnight.

Biochemistry techniques

2.15 RNA/Protein extractions

RNA and Protein was obtained for a 1 cm^2 leaf disk, ground on liquid nitrogen, using the nucleospin RNA/Protein kit (Macherey-Nagel), and prepared according to the protocol.

2.16 Protein preparation

The protein samples were heated for 10 mins AT 70 ^oC to ensure the correct migration on the gel.

Polyacrylamide gels were produced as follows:

Resolving gel: 3.8 ml of 1.5 M TRIS-HCl (pH 8.8), 6 ml of acrylamide (30% w/v acrylamide 0.8 % w/v bisacrylamide, 1.5 ml of 10 % SDS and 3.7 ml of H_2O. Polymerization was achieved through the addition of 150 µl 10% ammonium per sulfate (APS) and 6 µl tetraacetylethylenediamine.

Stacking gel: 1ml of 1M tris- HCl (pH 6.8), 1.3 ml of acrylamide-bisacrylamide mix, 5.4 mL of H_2O, 800 µl 10% SDS. Polymerisation was achieved through the addition of 150 µl 10% ammonium persulfate (APS) and 6 µl temed.

2.17 SDS-PAGE and protein transfer

Soluble proteins were separated on 10 % SDS-polyacrylamide (SDS-PAGE) gels (Laemmli, 1970) at 60 V through the stacking portion of the gel and then at 120 V through the separation portion of the gel (at room temperature). Samples were loaded on an equal protein level. The running buffer contained 25 mM tris-HCl 192 mM glycine and 0.1 % (w/v) sds. The gels were removed from the housing and proteins were subsequently transferred onto Immobilon PVDF membrane (Millipore, Bedford, UK) using Mini Trans-Blot Electrophoretic Transfer Cell (BIO-RAD) at 66 V for 60 minutes the transfer buffer

contained 25 mM TRIS-HCl, 192 mM glycine and 20 % (v/v). an ice block was included in the tank to keep the buffer cool.

2.18 Immunoblotting

Once the transfer was complete the immunoblotting follower several steps. The PVDF membrane was blocked with 6 % (w/v) powered milk (Marvell) mixed into phosphate buffered saline (PBS (137mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH_2PO_4)). The membranes were washed with PBS-Tween 20 (PBST (0.05 % v/v)) twice for 30 minutes. The membrane was the incubated with primary antibody contained in a 3 % (w/v) PBS solution, the incubation lasted a minimum of 1 hour but could be left over night. After the incubation the membrane was washed for in PBST three times for 30 minutes each time. The membrane was then incubated in the secondary antibody solution, containing the secondary conjugated antibody (1:2000) in a skimmed milk powder (3 % w/v) PBS solution for 1 hour. The membrane was then washed with PBST three times for 30 minutes each time.

The membranes were placed on a clear plastic sleeve and soaked with an enhanced chemiluminescence (ECL) solution (Amersham) for 2 minutes. The excess solution was removed and imaged.

Photosynthetic and phenotypic measurements

2.19 Gas exchange analysis

All gas exchange analysis was conducted with the Li-COR 6400 (LI-COR Biosciences, NE, USA) infrared gas analyzer (IRGA). The IRGA was calibrated before use, using a known CO_2 standard (Linde Gas Ltd, Stratford, London, UK). The measurements were taken at a CO_2 concentration of 400 ppm and a saturating light level of 2200 μmol m^{-2} s^{-1}, unless otherwise stated. The vapour pressure deficit (VPD) was maintained to as close

to 1 kPa as feasibly possible in the field conditions by adjusting the temperature to 2 ^{o}C above the ambient temperature, and fine tuning the moisture levels via the desiccant humidity controls. The levels of blue light were kept at 10 % and the flow was set to 300 μmol m^{-1}.

2.19.1 Light response curves (*AQ*)

Light response curves measured the carbon assimilation (*A)* of the plant as a function of changing irradiance levels. The plants were clamped in the IRGA and left to stabilise at a light level of 2200 μmol m^{-2} s^{-1} and a CO_2 concentration of 400 ppm, the CO_2 concentration was kept constant throughout the curve. Assimilation, stomatal conductance and fluorescence data was measured at decreasing PPFD levels. The PPFD levels for the light curves were as follows: 2200, 2000, 1700, 1400, 1100, 800, 600, 425, 250, 150, 100, 50 and 0 μmol m^{-2} s^{-1}.

2.19.2 CO_2 response curves (*A/C$_i$*)

The plants response of *A* to changes in intercellular CO_2 was measured. Light levels were set to saturating and kept stable throughout the curve (2200 μmol m^{-2} s^{-1}). The reference CO_2 concentrations were set to a program of 400, 250, 150, 100, 50, 400, 550, 700, 900, 1100, 1300 and 1500 ppm.

2.20 Field trial design and harvest measurements

The location of the field trial was at the University of Illinois Energy Farm field station (40.11°N, 88.21°W, Urbana, IL, USA). The field trial site was prepared in a similar manner each year. Two weeks before the seedlings were due to be transplanted, the field site was prepared via rototilling and harrowing. The pesticide chlorpyrifos (1.5 g m^{-2} Lorsban 15G Insecticide, Dow AgroSciences Canada Inc., Calgary, Alberta, Canada) was worked into the field at this time to protect the plants from cut worms (*Agrotis ipsilon* and *Peridroma saucia*).

Sulfentrazone (29 µL m^{-2} Spartan 4F pre-emergence herbicide, FMC Agricultural Solutions, Philadelphia, PA, USA) was applied to the field to control weed populations and at the same time the site fertilised with a slow-release fertiliser (30.8 g m^{-2} ESN Smart Nitrogen, Agrium US Inc., Denver, CO, USA)

For each field trial the seeds were germinated in climate control chambers with a 16 h photoperiod at 24 ºC. In the 2016 season the seedlings were moved into individual pots of 100 ml. In both the 2017 and 2018 trials the seedlings were moved into hydroponic trays (Trans- plant Tray GP009 6 9 12 cells; Speedling Inc., Ruskin, FL). The soil used was specialist soil designed for hydroponic agriculture (Pro-mix PGX, Premier Tech, Quakertown, PA, 248 USA) for growth after germination. Six and nine days after the seedlings were transplanted to the hydroponic trays Mancozeb (Dithane Rainshield Fungicide at 1g L-1, Dow AgroSciences Canada Inc., Calgary, Alberta, Canada) was added to prevent foliar fungal disease. Etridiazole fungicide (Terramaster 4EC to a final concentration of 78 µl L-1, Crompton Manufacturing Company Inc., Middlebury, CT, USA) was added 5-7 days after the seedlings were transplanted. Etridiazole protects the plants from root fungal diseases. Whilst the seedlings were growing in the hydroponic trays, the water was fertilised with 20-10-20 water-soluble fertilizer (Jack's Professional, JR Peters Inc., Allentown, PA, USA). The concentration of the fertiliser was checked with a total dissolved solids meter (COM-100, HM Digital Inc., Culver City, CA, USA) and adjusted to 100 ppm accordingly.

Plants were transplanted to the field after around 18 days in the individual pots/hydroponic trays. The plants were spaced at 30 cm and the blocks were spaced 75 cm apart. To reduce edge, affect a boarder of wildtype plates were used as the outside plant of each block, the entire experiments were surrounded with an additional two rows of boarder plants. Watering was provided every other day or daily when gas exchange

measurements were being conducted, in 2016 this was completed with rain towers and in 2017 and 2018 drip irrigation was used as described by (Kromdijk et al., 2016). The plants were harvested and left to dry in a custom-built drying oven at 60 ^{o}C.

For all experiments, measurements were taken for leaf area (LI-COR LI-3100C), leaf number, and stem length at the time of harvest. Plant matter above the soil line was harvested and separated into leaf and stems for drying and weighing.

Due to regulations of the permits obtained for the growth of the transgenic plant, all plants needed to be harvested before seed was produced. As such plants were harvested no later than when the first seed pod was formed.

2016: The seed were germinated and after 11 days moved into individual pots (350 ml). The plants grew in the glasshouse for a further 15 days before being moved in to the field. The plants were allowed to grow for a further 14 days before being harvested.

2017: The plants were germinated in growth chambers and after 12 days were moved into hydroponic growth trays (Trans-plant Tray GP009 6912 cells; Speedling Inc., Ruskin, FL) where they grew for a further 20 days before being moved in to the field. The plants were harvested after approximately 30 days.

2018: The plants were germinated in growth chambers and after 12 days were moved into hydroponic growth trays (Trans-plant Tray GP009 6912 cells; Speedling Inc., Ruskin, FL) where they grew for a further 16 days before being moved in to the field. There were five individual harvest points to the 2018 experiment, the first two being from the glasshouse 20 and 28 DAS. The remaining three being form plants in the field 47, 55 and 62 DAS.

2.20.1 Experimental design – 2016

The field trials were planted to a replicated control design. The trial in this study has 7 individual rows of 4x14 plants, including 2x12 experimental plants and a wildtype border to normalise edge effect. Of the seven rows, row 1, 3, 5 and 7 were control blocks and row 2, 4 and 6 were S_B, *C6* and H-protein respectively. Each manipulation used two independent lines. The control plants used wildtype plants and a mixture of azygous plants.

2.20.2 Experimental design – 2017

Both experiment in 2017 were built to an incomplete block design. The experiments had 18 blocks divided into 6 rows of three blocks. There was a complete set of genotypes in each row, and one genotype per manipulation in each block. The genotypes were assigned using a random number generator.

2.20.4 Experimental design – 2018

The 2018 trial was set up as a complete random block design, with three harvest points. The 6 of the 18 blocks were randomly selected for each harvest. Each block was 6x16 plants, spaced 30cm apart. The central 56 plants were divided into 14 rows of 4 plants per genotype. The outer plants of each block were boarder plants.

2.21 Statistical analysis

All data analyses were completed in SPSS statistics (IBM Corp. Released 2017. IBM SPSS statistics for Macintosh, Version 25.0. Armonk, NY: IBM Corp), and R (https://www.r-project.org/). 2016 biomass harvest data was tested for homogeneity using the Brown-Forsythe test and normality was determined using Shapiros-Wilks test, which was followed with a T-test. Harvest data from 2017 and 2018 was compared using linear mixed model, using lmer function and type III ANOVA.

Chapter 3 – Production and Identification of Homozygous Transgenic Tobacco Plants

Introduction

Producing plants that have targeted manipulations to the genome, using a transgenic approach, is useful in order to understand how photosynthesis can be improved, and what impact increasing primary metabolism can have on productivity. The advantage of this approach is that phenotypic changes resulting from these manipulations can be measured in vivo, and ideally this work would be carried out in the targeted crop plants. Due to species-specific difficulties with the transformation process, it can be both time consuming to produce plants with targeted manipulations and to get the number of seeds needed for large phenotyping experiments. One approach is to use a model species such *Nicotiana tabacum* that can be easily transformed yet can also be grown as a crop. *Nicotiana tabacum* is a dicotyledonous, amphidiploid plant, bred from the cross of *N. sylvestris* and what is believed to be *N. tomentosiformis* (Kawashima et al., 1976). There are multiple cultivars of *N. tabacum* that are used in scientific research, and although they are the same species, each cultivar has their unique properties. *N. tabacum*. cv. Petit Havana (P.Havana) has a lifecycle of 3 to 4 months from seed to seed. P. Havana grows to between one and two meters and produces thousands of seeds per plant. Overall, these traits provide the basis for a good model specie. *N. tabacum* is very easy to transform in comparison to other species, i.e. rice species, and by being an amphidiploid, homozygous seed it can be produced from the T1 generation.

Agrobacterium-mediated transformation is one of the most commonly used processes for the production of transgenic plants including *N. tabacu*m. The bacteria used in this process, *Agrobacterium tumefaciens*, is a soil-based plant pathogen that naturally infects dicotyledonous plants by entering wounds and causing formation of crown gall tumours (Smith and Townsend, 1907, Anderson, 1979). *A. tumefaciens* transfers the T-DNA

section (T-region) of the tumour inducing (Ti) plasmid into the nucleus of the infected organism. Once inserted in the host genome, it is transcribed, which is the cause of the crown gall disease (E W Nester et al., 1984). There are two categories of genes in the T-DNA of *A. tumefaciens*. The first set produces the hormones necessary for tumour growth (auxins and cytokines). The second set of genes produces opines. These are used by the bacteria for the source of carbon and nitrogen. The genes are necessary for the insertion of the T-DNA reside in the Ti vector (~35 grouped virulence (*vir*) genes). These will insert all the DNA between the left and right border of the T-DNA and into the host plant, irrespective of the genes in this T-DNA region (Hooykaas et al., 1984, Stachel and Zambryski, 1986). The *vir* genes and the T-DNA do not necessarily need to be on the same plasmid. In order to utilise this, scientists have disarmed the Ti vector and designed innate vectors that have the T-DNA borders that will be targeted by *vir* genes from specific Ti plasmids. By having the ability to insert genes in these designed constructs it is possible to deliver a selected DNA sequence into the plant. The technique of using agrobacterial mediate transformation process does have some limitations as the T-DNA insertion happens at random and is not targeted to a specific location within the genome, which can cause problems by disrupting a gene coding sequence.

Several potential targets for manipulation of photosynthesis have been identified, whether these were introduced from foreign species or the upregulation of natively expressed proteins. This project will focus on three individual genes as single manipulations and in combination. A cyanobacterial bifunctional gene (S_B) with a dual function catalysing the reactions performed by both FBPase and SBPase in the C3 cycle, was introduced to work in conjunction with the native SBPase in the plant to increase its enzymatic activity. The S_B gene was selected due to the overexpression of SBPase producing higher biomass model species (Lefebvre et al., 2005, Simkin et al., 2017) and, when overexpressed in

wheat, produced higher grain yielding plants (Driever et al., 2017). The S_B gene functions as SBPase (Miyagawa et al., 2001) and as this is from a foreign organism, there is very little homology with the native genes. As a consequence, this reduces the likelihood of the gene being subject to co-suppression by the plant. Due to the nature of the S_B gene, it will also catalyse the dephosphorylation of fructose-1, 6-bisphosphate (F-1, 6-BP) to fructose 6-phosphate, though this is not deemed to be a rate limiting step under normal growing conditions.

The second gene to be introduced, cytochrome *c*$_6$ (*C*$_6$), codes for a protein that functions in its native, red algal as an alternative to plastocyanin (Katoh, 1960, Katoh and Takamiya, 1961). Cytochrome *c*$_6$ transfers electrons between the cytochrome *b*$_6$*f* complex and PSI and has a similar redox potential to native plastocyanin (Ullmann et al., 1997). Some cyanobacteria and green alga express this protein as a replacement to the copper containing plastocyanin when the organism is under copper deficiency stress (Merchant and Bogorad, 1987). Expressing cytochrome *c*$_6$, in the model *species Arabidopsis thaliana* was shown to improve photosynthetic rates, carbon assimilation and led to improved growth. This was achieved while indirect measurements of the plastoquinone pool showed a decrease of around 30 % (Chida et al., 2007). Cytochrome *c*$_6$ functions in conjunction with the native plastocyanin to increase the rate of electron transfer through the ETC, boosting productivity.

As well as *C*$_6$ and S_B being expressed, the H-protein of the glycine cleavage system (GCS) was overexpressed. This is a complex of interacting proteins comprising of three enzymes (P-, T- and L-protein) and the H-protein. The CGS is extremely important to the metabolism of glycine to serine in the photorespiratory pathway and although the H-protein has no catalytic activity, it functions as the communicator or “linking” protein

between the three enzymes of the GCS, interacting with each enzyme in turn via the lipoylated arm of the protein. Overexpressing just the H-protein has been demonstrated to boost the rate of the entire GCS (Hasse et al., 2009). When the H-protein is overexpressed in *Arabidopsis thaliana*, it led to higher carbon assimilation and an increase in biomass (Timm et al., 2012).

The three proteins that have been selected for the project all target different pathways within photosynthesis. The overexpression of the H-protein of the GCS aims to increase the rate at which carbon is metabolised through photorespiration, providing carbon back into the C3 cycle. The expression of the bifunction FBPase/SBPase gene has been shown to boost the rate of RuBP regeneration. Additionally, introducing cytochrome c_6 has demonstrated the potential to increase the availability of chemically stored energy to these processes by increasing the flow of electron through the electron transport chain.

Overall, the aim of the work was to produce transgenic lines expressing the H-protein of the glycine cleavage system, cytochrome c_6 and the bifunctional FBPase/FBPase. These proteins will be expressed both singular, in double and triple combinations. The transgenic plants will be phenotyped in field conditions for improvements to carbon assimilations and whether this impacts the growth and final biomass of the plants.

Results

3.1 Tobacco transformation and selection pipeline

The aim of the work presented here was to produce T2 homozygous plants, expressing the inserted genes with a stable genotype (Fig. 3.1). In order to achieve this, 100 leaf discs were incubated with the agrobacterium strain (LBA4404) containing the gene of interest. Leaf discs were then placed on selective media and emergent shoots were cut and transferred to rooting media. Suitable plant shoots normally started appearing at 71 (+/-3.5) days after being transformed, and a further 69 (+/-15) days were needed to produce 50 shoots from separate callus. Once the putative transformants had an established root system, molecular analysis was conducted, and the plantlets transferred into soil. Samples were analysed to confirm that the DNA was present, the DNA was transcribed and how many insertion events happened during the transformation. The number of transgene insertion events was determined at this stage via iDna Genetics Ltd (iDna) and the T0 plants were grown in glasshouse conditions and left to self-fertilise. Once they had been moved into soil, seeds taken from these plants were dried down in a custom design humidity-controlled chamber for seven days to provide equally dried seeds. The methodology undertaken to determine the transgene insertion events is described by Bartlett et al. (2008). To produce homozygous T2 seed, T1 plants was grown under glasshouse conditions and sampled for molecular characterisation and transgene number. Plants were selected on the three main characteristics:

1) The zygosity: as identified from analysis of data provided by iDna, where a plant will be kept only if it has segregated into a null sergeant (azygous), hemizygous and homozygous plant.

2) Molecular characterisation: Any plant that does not show an increase in transcript and had a visible increase in protein (unless a null sergeant) will be disposed of.

3) Growth phenotype: Due to the often-malformed nature of plants coming from tissue culture, this is the first stage where it is possible to get an accurate representation of the plants' growth, and whether the transgene had been incorporated into a detrimental place within the plant's genome. Plants that exhibited a strange growth pattern are removed at this stage.

By using these three methods of selection, it is possible to produce genetically stable T2 seed that should have expression of the gene of interest. Wildtype seeds were grown in tandem with the T1 seed, so true controls of wildtype and azygous plants were used phenotyping experiments.

The total time that is needed to produce genetically stable T2 seed ranges from 1 to 1.5 years.

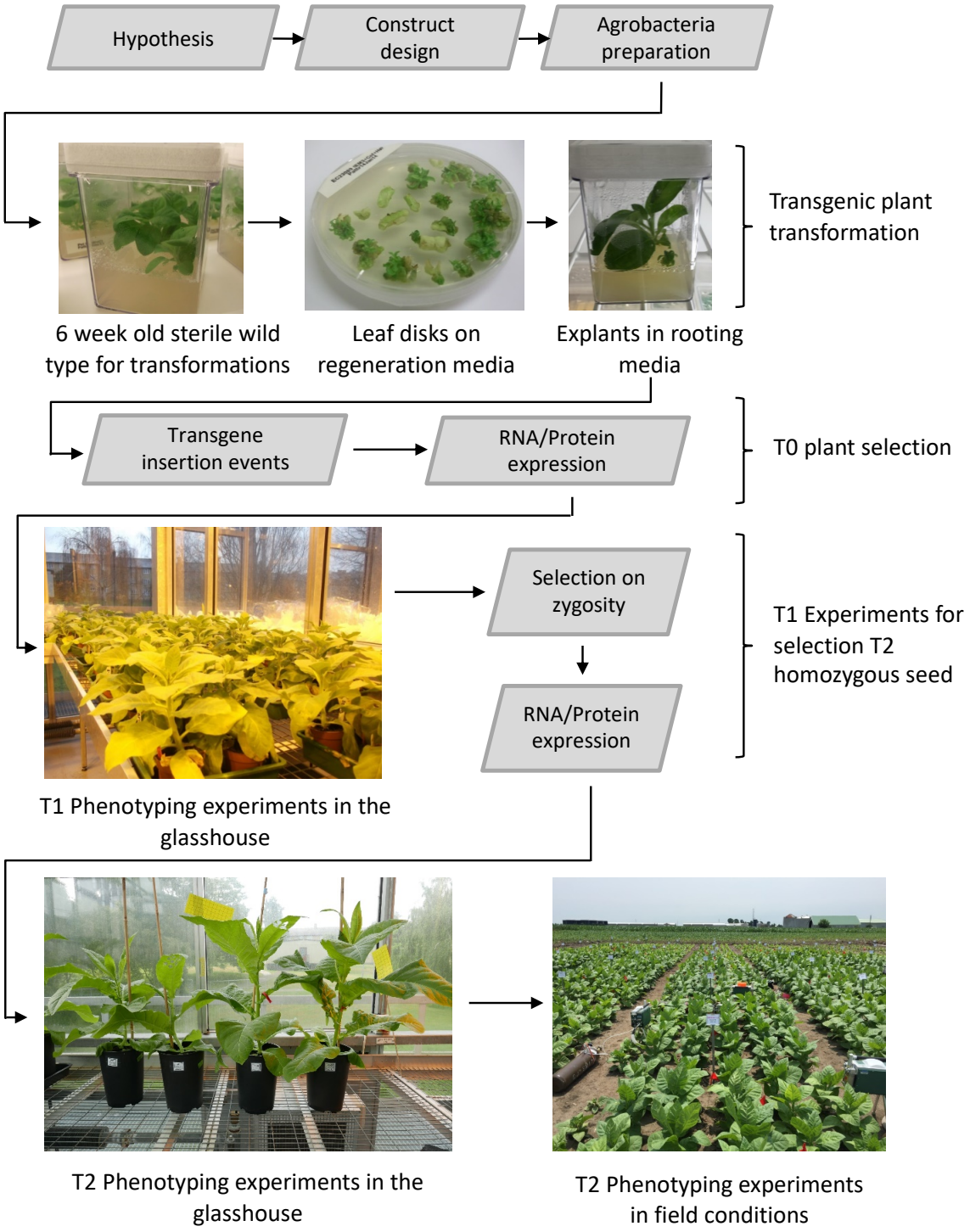


Figure 3.1. Flow diagram representing the pipeline for producing transgenic tobacco plants, from leaf disks to field trials. The pipeline needed to produce transgenic plants suitable for field trials. This project stared at the Agrobacterial preparation stage, and this was used to infect 6-week-old leaf disks. The total time taken for transgenic plant transformation phase of the pipeline was between 3-5 months. From transforming leaf disks to having T2 seed prepared for glasshouse trials ranged vastly but was on average around 8 months.

3.2 Transformation efficiency

Each construct was transformed into 100 leaf disks. From each independent transformation event, four plantlets were moved into rooting. At 50 independent events the remaining leaf disks were disposed of. Four plantlets were moved to increase the probability of rooting by reducing the chance of death by mishandling. Once in the rooting media, the survival rate was measured based on three different grades. Here, ~18 % of the explants did not survive in rooting media, while ~10 % either died in soil or did not produce seed, and ~73 % of primary transformants survived to produce T1 seed (Fig. 3.2, Table 3.1).

3.3 Transfer DNA insertion events per primary transformant

The number of transgene insertion events was analysed by iDna Genetics Ltd following the method described by Bartlett et al. (2008). Analysis of >900 plants provided a large set of data that demonstrated how common multiple insertion events are in *N.tabacum*. The analysis identified the most common outcome to have a single insertion event, this was the case >66 % of the time. And each additional T-DNA copy becomes less common with two, three and four insertion events occurring 16 %, 6.5 % and 4.4 % of the time, respectively (Fig. 3.3).

3.4 Segregation ratios

The segregation ratios of the T-DNA inserts, from progeny from the original transformants, was 0.22:0.54:0.24 (Table 2) for azygous:hemizygous:homozygous plants. This, when keeping with the expected ratios of 0.25:0.5:0.25 for an amphidiploid plant. There were 335 T1 plants used in this analysis and this T1 plants were progeny of 35 independently segregating lines, from five different constructs.

Construct	Dead in Rooting media (%)	No seed (%)	Seed producing line (%)
EC23083	34	8	58
EC23084	16	8	76
EC23085	20	4	76
EC23086	10	10	80
EC23089	22	2	76
EC23088	32	28	40
EC23025	12	10	78
EC23028	6	16	78
EC23030	6	0	94
Mean	17.6	9.6	72.9
Standard error	3.5	2.8	5.1

Table 3.1. The survival efficiency of explants put into rooting media. A Sub section of transformed constructs are presented to show the survival rate of explants from when they have been put to rooting media. The total number or plants per construct was between 50-60 and are presented as percent of total plants put too rooting media. The constructs show a large range of successfully transformed, seed producing plants, from 40-90 %, but with a mean seed yield of 72.9 % of plants. 17.6% of trans formants died in rooting media and 9.6 % didn't give seed after being moved to soil, meaning 72.9 % of explants put to rooting media produced viable seed. See appendix figure 1.2 for the full list of constructs.

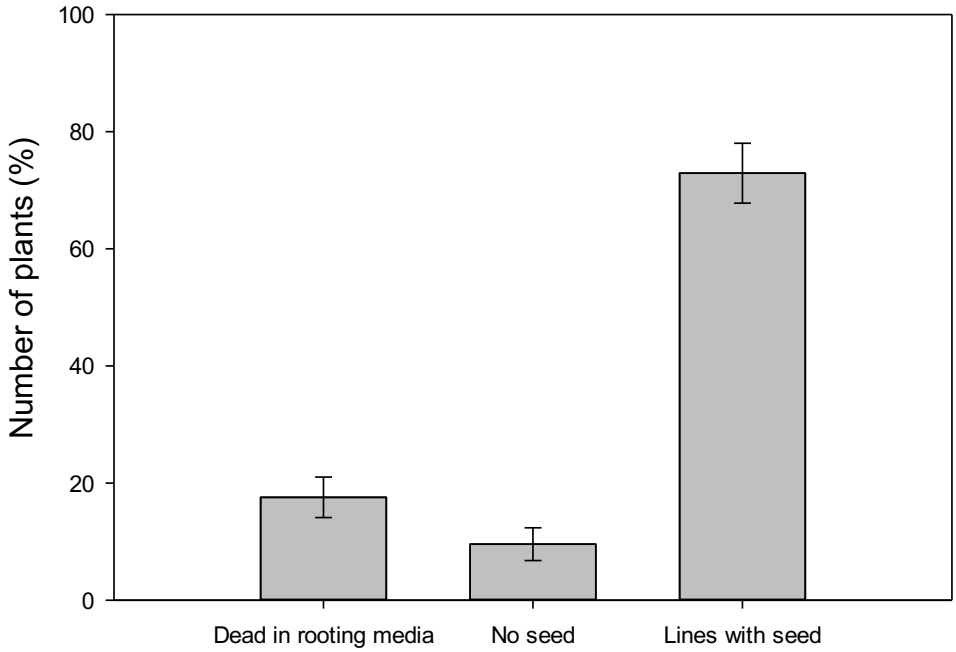


Figure 3.2. The survival efficiency of explants. put into rooting media. A Sub section of transformed constructs are presented to show the survival rate of explants from when they have been put to rooting media. The total number or plants per construct was between 50-60 and are presented as percent of total plants put too rooting media. The constructs show a large range of successfully transformed, seed producing plants, from 40-90 %, but with a mean seed yield of 72.9 % of plants. 17.6 % of trans formants died in rooting media and 9.6 % didn't give seed after being moved to soil, meaning 72.9 % of explants put to rooting media produced viable seed. See appendix figure 1.2 for full list of constructs. Error bars represent standard error.

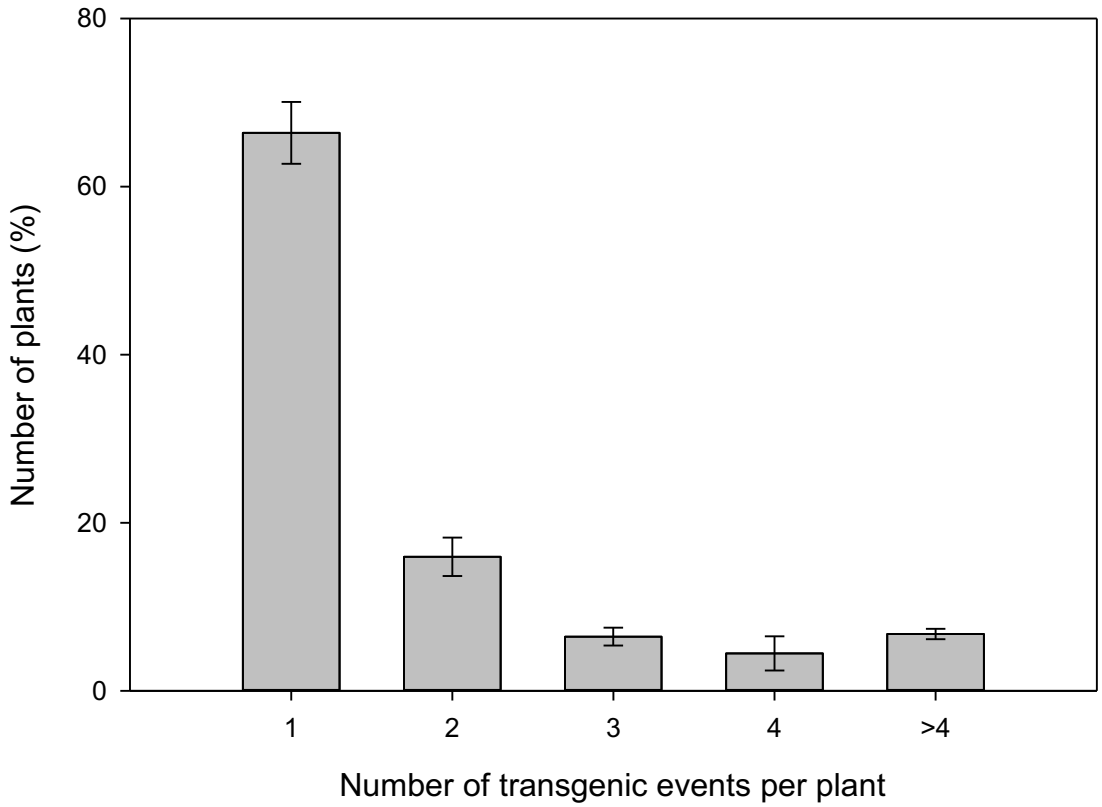


Figure 3.3. Transfer DNA copy number determined via quantitative RT-PCR of the hygromycin b gene. The number of transgene insertion events in each plant was measured by iDna genetics (iDna genetics ltd, Norwich) using qPCR. The number of Hygromycin B genes in each T0 transgenic plant was used to determine the number of transgene insertion events. >900 transgenic plants from 22 constructs are displayed. 66.4 % of plants contained 1 transgene, 16 % of plants contained two transgenes and 6.4 % contained three transgene insertions. Error bars represent standard error.

Construct ID	Number of plants	Ratio of segregation of transgenes in plants					
		0 (+/- SE)		1 (+/- SE)		2 (+/- SE)	
EC23239	110	0.22	0.03	0.53	0.06	0.25	0.05
EC23131	50	0.22	0.06	0.66	0.08	0.12	0.05
EC23140	49	0.24	0.11	0.49	0.06	0.27	0.08
EC23132	53	0.19	0.03	0.49	0.06	0.28	0.05
EC23027	73	0.22	0.05	0.59	0.04	0.19	0.05
All	335	0.22	0.02	0.54	0.03	0.24	0.03

Table 3.2. The segregation ration of T1 plants from 5 sets of transformations. The ratio of segregating plants from 335 segregating plants is 0.22:0.54:0.24 . These plants were from three separate T1 phenotyping experiments and 35 independently segregating lines were used in the analysis. See appendix figure 1.2 for a full breakdown of constructs.

3.5 RNA expression

qPCR was used to detect transcript levels of the introduced genes. T0 plants that were transformed with the S_B gene had a range in transcript levels (Fig. 3.4), but the transformed plants that had multiple insertion events do not appear to have more transcript levels that the single insertion T-DNA gene plants (Fig. 3.4). In subsequent generations, a pattern of increased transcript levels from T1 homozygous plants to T2 homozygous plants is present (Fig. 3.5). A difference between T1 hemizygous plants and T2 hemizygous plants may also be apparent. The transcript levels for T1 plant does not seem to relate to the transcript levels in the T0 plants (Fig. 3.5).

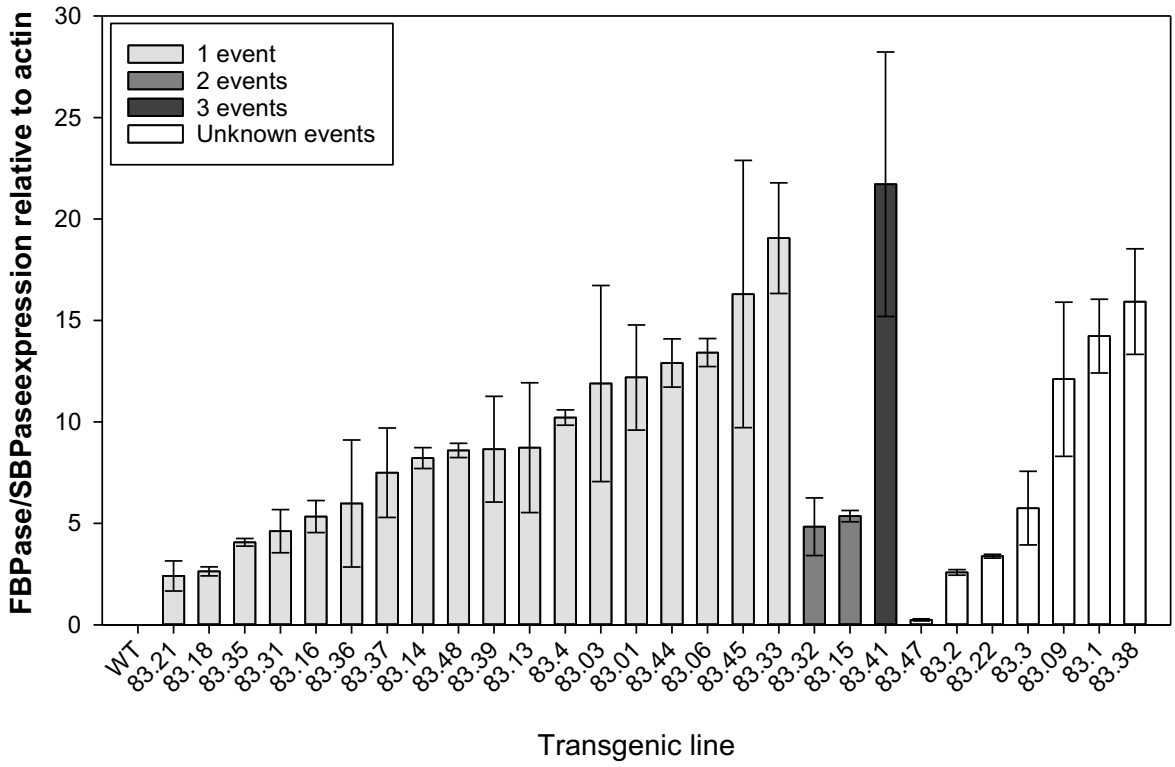


Figure 3.4. RNA transcript levels of a range of T0 S_B lines. qPCR showing transcript levels of the introduced S_B gene relative to the actin. Plants were sampled as they were taken from the rooting media and placed in to soil. The range of transcript levels varies from around 2.5-fold of actin to 19 fold of actin for the single inserting plants. Light grey bars represent plants containing a single transgene insertion, the slightly darker bars represent two insertion events, the dark grey bar represents three insertion events and the white bars are representing plants that have an unknown number of transgene insertion events. The error bars represent standard error.

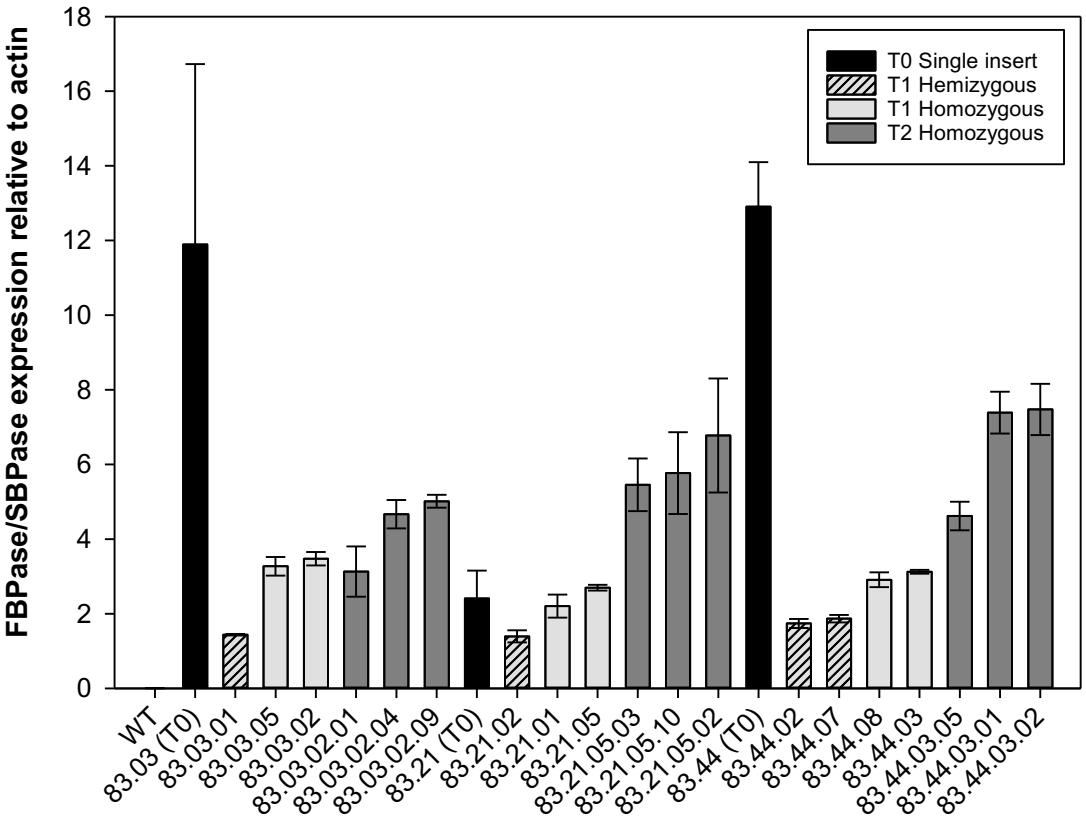


Figure 3.5. RNA transcript levels of T0 lines and there T1 and T2 progeny. qPCR showing transcript levels of the introduced S_B gene relative to the actin. T0 lines were samples taken as plants were moved from rooting media, T1 plants were taken from the youngest fully expanded leaf during the T1 phenotyping experiment.

Discussion

The ability to evaluate the impact of the manipulation of genes in the photosynthetic pathways, and how this translates to improved growth over the lifecycle of the transgenic plant, can be achieved by producing stable transformations expressing the gene of interest. Studies that are designed for glasshouse phenotyping experiments have had the ability to individually screen each plant for transcripts to confirm the presence of the gene of interest. If a plant does not have the gene transcript present, i.e. through segregation, it is simple to remove and replace them. The transgenic plants created for the following experiments are to be phenotyped in large scale field trials. Due to the size of these trials, it is not feasible to screen individual plants before committing them to the experiment. This makes it essential to produce plants that have a stable, homozygous genotype, thus avoiding issues with segregation in the field trials. This study has looked at streamlining the transformation process to produce genetically stable plants, appropriate for large scale field trials.

The most efficient way to produce genetically stable transgenic plants is to use T0 transformants that have a single gene insertion event. By only using transgenic plants with single insertion events, and given the amphidiploidy nature of *N. tabacum,* the progeny from self-fertilisation will segregate as a diplody organism. Hence, the possible zygosity of the T1 seed outcomes will be azygous, hemizygous and homozygous in the ration 0.25:0.5:0.25 respectively. Therefore, it is possible to obtain T2 seed that will have a stable genotype. The segregation ration that was obtained from 335 independent transformations was 0.22:0.54:0.24 (Table 2). The ratio that was obtained from the transformations is extremely close to the predicted ratio and show firstly the plants a reproducing correctly and that it is possible to archive T2 homozygous seed.

The feasibility of only using plants with single T-DNA insertion events relies on having a methodology identify them. The insertion events in each transgenic plant were determined via iDna Genetics ltd, as explained by Bartlett et al. (2008). The results of analysing >900 primary transformants revealed how 66.4 % of transformants were of a single insertion event. The importance of this is from the 50 independent events that were obtained from each construct, on average >30 are likely to have a single insertion, and so suitable for taking forward with in the experiment. In previous field trials, manipulations of photosynthesis produced 30 independent lines that were subsequently reduced to three, depending on the phenotype exhibited (Kromdijk et al., 2016). As there is over a 60 % chance of single gene insertion during the transformation process, 50 independent lines should be sufficient in producing enough plants for phenotyping experiments.

The transcript levels for each independent T0 line were checked to confirm the gene had been inserted and functioning. The qPCR results of the T0 plants (Fig. 3.4) provided evidence for a large range of in transcript. This range of transcript is not surprising due to the sampling time and growing conditions prior to the sampling. T0 plants are samples after being removed from rooting media. This means that plants are sampled on different days and at different development stages. This can be seen in the transcript through the subsequent generations (Fig. 3.5). The level of transcript for plants expressing the bifunctional gene is extremely varied at T0 stage, ranging from 2 to 14 times the relative actin transcript level. Interestingly, at the T1 (homozygous) generation the range is between 2 to 4 times actin, showing a much more similar range, and one that does not necessarily correlate with the T0 levels.

The relative transcript levels of the bifunctional gene vary between the T1 and T2 generations and even with in the T1 generation for plants that have a single of a double

copy of the gene. The trends seem to show an increase for T1 homozygous over T1 hemizygous plants, and transcript levels between T1 and T2 homozygous also differ, with the T2 plants seemingly having higher transcript levels. It is necessary to measure the transcript levels at each generation of the transgenic line, as it has been demonstrated that *N.tabacum* plants are susceptible to post-transcriptional silencing of introduced genes, throughout the generations (Elmayan and Vaucheret, 1996). Determining generation effects transcript levels would provide insightful information on the stability of the introduced gene through the generation (Fig 3.5), however, as the different generations were sampled from different experiments, with differing growing conditions, it is not possible to directly compare or correlate the samples. To achieve a rigorous comparison the plants would need to be grown together in the same conditions and sampled at the same time.

Goldengate cloning was used to produce the plasmids used in the transformation event. This method of cloning allowed the construct to be designed as so the selective marker enters the genome last (Engler et al., 2014). Having the selective marker enter the genome last reduces the chance of false positives from partially inserted plasmids. As this part of the T-DNA enters the genome last, it was the part of the plasmid that was used to determine the number T-DNA insertion events in each transgenic plant. The methodology used to assay the plants (Bartlett et al., 2008) provides a high level of accuracy in determining the T-DNA insertion events, and this is evident with the expected segregation ratios. The predicted segregation ratios, for a single T-DNA insertion transgenic plants, are: 0.25:0.5:0.25 for 0:1:2 transgenes, respectively, for a self-fertilised plant. The ratio that was obtained from the segregation of transgenic plants was 0.22:0.54:0.24. The discrepancies between the expected and obtained ratios were minimal but can still be explained. There could have been an unconscious bias when

pricking out seedling from germination pots with larger seedling being selected. Due to the manipulations, this could possibly contribute to a selection of the transgenic plants over the null segregates. It is possible that some plants were negatively impacted from expressing the transgene, which will also distort the ratio. The overexpression of the H-protein, under a constitutive promoter leads to homozygous plants dying (Lopez-Calcagno et al., 2019), again this will distort the ratio.

The data supplied by iDna Genetic ltd, on the transgene insertion number provides a high level of precision. This is demonstrated by the plants segregating from single T-DNA inserts. However, the resolution of the assay becomes diminished when distinguishing between plants with many transgene copies. This is challenging when selecting for crosses. The selection marker for the S_B, *C6* and H-protein plants all use the hygromycin b resistance gene as a selective marker and so having a homozygous cross of these three individual insertions will tally to a total of six T-DNA insertions.

The methodology that is implemented to perform the assay loses resolution as more copies of the gene are present, and this is problematic for determining the T-DNA insertion number in plants that were made via crossing. The cross of the S_B, *C6* and H-protein line will have a total of six T-DNA insertion events once it is at a homozygous state for all three genes. Given the lack of resolution in the higher copy numbers, it makes the selection of crosses problematic as false positives can lead to an unnecessary use of extra time and resources. There are ways to overcome this issue. First, it would be possible to produce probes for individual genes in the constructs and not just the selection markers. At the T0 stage this would not be a sensible option as it would be possible to detect incomplete T-DNA insertion events as false positives. If the original T0 plant has the selective marker probed for, while the future generations only feature the gene of

interest, this would not be an issue. The drawback of this approach is the major cost and how this cost could potentially be tripled by having to perform three assays on each sample (i.e. triple cross) rather than one. It would be possible to select for homozygous crosses with selective plates and PCR. However, with a segregation ratio 63:1 for obtaining a homozygous plant, this method requires significant time and space into the selection process, which is not an issue when outsourcing the assay.

The most cost-efficient route to obtain homozygous plants would be to create new constructs that contain all the desired genes and thereby transform a new set of plants. However, there are positive aspects to the crossing of transgenic plants. In fact, the time that is required to produce a new manipulation is equivalent to one lifecycle, around three months. This is considerably less time than what is required under production via agrobacterial transformations, as previously described (Fig. 3.1). Another benefit of the crossing plants over agrobacterial transformation is the ability to select plants that have had the original construct evaluated, both at a molecular and physiological level. Crossing from previously made transgenic plants provides the opportunity to run experiments with direct controls for each gene that has been introduced. For example, after crossing individually phenotyped lines from two different constructs it is possible to evaluate if the new transgenic plant will have a synergistic, culminative or no increased phenotype over the parental lines. Having the previous generations act as a control mean the crosses can be more accurately judged over single gene manipulations. For this to be achieved the single gene manipulations and the parental lines, must be phenotyped. The following chapter will provide evidence of the phenotyping under the field conditions.

Chapter 4 – Preliminary field experiments to identify potential transgenic lines for fully replicated field trials (2016)

Introduction

Previous experiments have demonstrated that overexpression of proteins and enzymes involved in the photosynthetic process either individually or in combination can provide increases to productivity in the glasshouse environment (Simkin et al., 2015, Timm et al., 2012, Chida et al., 2007). Replicating these results in field conditions, however, has proved more challenging. Plants grown in natural conditions are subjected to a wider range of stresses and less ideal conditions compared to glasshouse grown plants. These include (but not limited to) less uniformed light levels, a more dynamic light profile, less abundant water, storm damage and pest damage.

It is rare for unsuccessful experiments to be published, hence the data on the number of unsuccessful field trials is limited. Yet, there are demonstrations of field trials that have shown the controls and transgenic plants performing equally. SBPase overexpressing plants were grown in environmental conditions, and also elevated CO_2 simultaneously. Rosenthal et al. (2011) grew tobacco plants overexpressing the SBPase gene and showed that under elevated CO_2 transgenic plants had a significant increase in the biomass. This phenotype was not present when grown under ambient CO_2, with transgenic plants not having a significantly different biomass to the controls. A more extensive trial, growing soybean expressing the bifunctional cyanobacterial gene, a protein performing as both FBPase and SBPase, had a comparable phenotype (Kohler et al., 2017). Transgenic and wildtype plants were grown over three growing seasons. The findings show is that under ambient CO_2 and temperature the increased yield phenotype was observed in only one of the three years, whereas for the soybean grown under elevated CO_2 and temperature the increase in yield was consistently observe in each of the three-years. The variation in the phenotype, in terms of yield, highlights the

importance of field trials to confirm the robustness of traits that have been observed in the glasshouse but also shows how changes in environmental conditions in subsequent years can have an impact on productivity.

Other notable field trials have produced promising results, manipulations to the rate of NPQ relaxation has provided a 15 % increase to biomass (Kromdijk et al., 2016), as well a Synthetic glycolate metabolism pathway, which acts as a bypass to photorespiration, providing biomass increases of up to 35 % (South et al., 2019). Both of these trails were conducted on *N.tabacum* cv Petit havana, demonstrating the ability of this plant as a model species for testing trait manipulations in field conditions.

The plants used in the experiments are transgenic lines with single gene manipulations. The endogenous gene for the H-protein was over expressed, as described by Lopez-Calcagno et al. (2019). Two foreign genes were introduced, a bifunctional FBPase/SBPase and cytochrome c_6 (Lopez-Calcagno et al., Unpublished). When these plants were phenotyped in glasshouse conditions, the plants exhibited an increase in dry biomass from 13 % to 38 % for the H-protein over expressers. Plants expressing S_B displayed an increase 35 %. The C_6 expressing lines were increased by 44 %. The aim of this chapter is to investigate whether transgenic plants grown in field conditions have a comparable phenotype to equivalent plants grown under glasshouse conditions.

Results

4.1 Construct generation

The full length coding sequence for the bifunctional FBP/SBPase (S_B) (slr2094), a codon optimised Cytochrome c_6 (C_6) (AFC39870) and the H-protein of the glycine cleavage system (AT2G35370) were used to generate entry vectors using Golden Gate cloning (Engler et al., 2009, Engler et al., 2008) (Fig. 4.1). These genes were under the control of FMV (Richins et al., 1987), CaMV 35s and ST-LS1 respectively and were used to transform *Nicotiana tabacum* cv. Petit havana. *Agrobacterium* mediated transformations were used to generate 50 independent lines per construct. To avoid co-suppression, the *Arabidopsis thaliana* H-protein gene was selected and as neither the S_B nor C_6 genes are native genes in tobacco, co-suppression was not a concern. Several lines were left to self-fertilise until the homozygous T2 generation and these transgenic plants were characterised for their molecular traits (Lopez-Calcagno et al., Unpublished, Lopez-Calcagno et al., 2019). Two lines per construct were selected for the field trials based on the experiments conducted in the glasshouse (Lopez-Calcagno et al., Unpublished, Lopez-Calcagno et al., 2019).

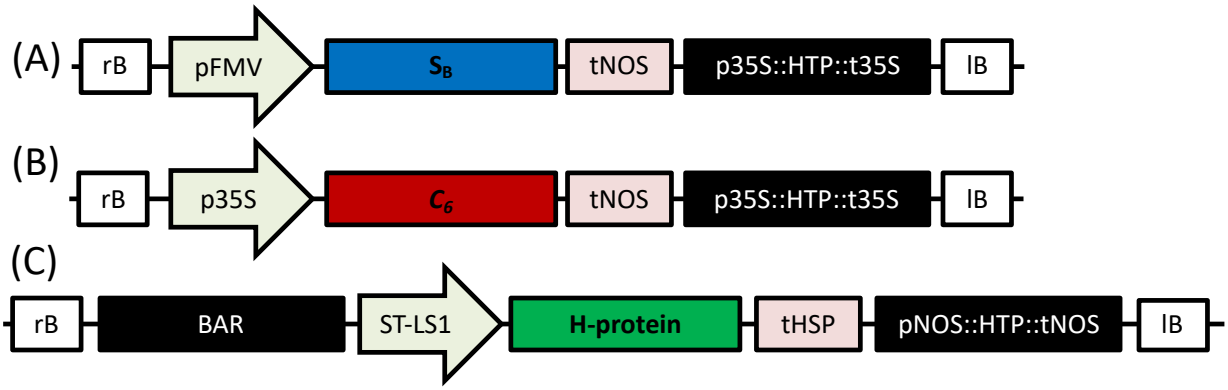


Figure 4.1. Schematic representation of the constructs used to transform *N.tobacum.* (A) Construct used to produce SB plants. (B) Construct used to produce C6 plants. (C) constructs used to produce H-Protein plants. LB/RB-T-DNA left/right boarder. p35S, cauliflower mosaic virus 35S promoter. ST-LS1, Solanum tuberosum ST-LS1 promoter. pNos, nopaline synthase promoter. pFMV, figwart mosaic virus promoter. BAR, Bialaphos resistance gene. HTP, hygromycin phosphotransferase gene. tNos, nopaline synthase terminator. tHSP, heat shock protein 18.1 terminator. t35S, cauliflower mosaic virus 35S terminator.

4.2 Field trials design

4.2.1 Field trial design-experimental design

The field trials were planted to a replicated control design (Fig. 4.2), which is an agricultural experimental design that is used to screen potential new varieties for increased yield and is based on differences between a treatment and a control. This experimental set up gives users the ability to include a large number of treatments in a relatively small space, but it cannot be used to directly compare treatments, nor does it provide robust statistical analysis of the results. The trial in this study had seven individual rows each of which was comprised of 2x12 experiment plants surrounded by a border (Fig. 4.2) The entire experiment was surrounded by a further two rows of wildtype plants to reduce edge effect and to protect against potential pests (Fig. 4.2).

One of the limitations of the replicated control experimental design is the contrast in microenvironments across the field, which means that the transgenic rows cannot be directly compared to each other. Analysis of the control rows that cover the full experiment provides evidence of the gradients of growth across the field. The data showed slight variation in the means for the measured parameters but there are no consistent trends in parameters measured between the different rows of control plants (Fig. 4.6) demonstrating that there was no observable positional affect within the experiment. The control plants used for the experiments included both wildtype and the azygous plants. The azygous controls are null sergeants taken at the T1 plant stage. There were no detectable differences between the two types of controls (Fig. 4.7), in terms of leaf area, height, stem length and biomass; demonstrating equal growth between all the control lines. As the azygous plants and the wildtype plants performance is similar this provides confidence that any differences observed in the transgenic lines are due to the changes

elicited by the introduction of the transgene and not somaclonal variation. In addition, the biomass results from the control block show that there are no observable positional effects across this field, and as both the wildtype and azygous perform equally the difference observed with the transgenic lines would be caused by the introduction of the gene (Fig. 4.6).

4.2.2 Field trials design-Experimental plants

The transgenic plants used in this experiment contained expression constructs consisting of single genes involved in photosynthesis (Fig. 4.1). These transgenic plans were T2 generation and had been selected from T1 plants with a single insertion event and were double homozygous. The wildtype control seed were selected from several plants grown in glasshouse condition, alongside the transgenic plants. The azygous controls were selected from the segregating plants at the same time as the T2 homozygous plants. Protein expression was checked via immunoblot, with custom raised antibodies on samples taken from the experiment.

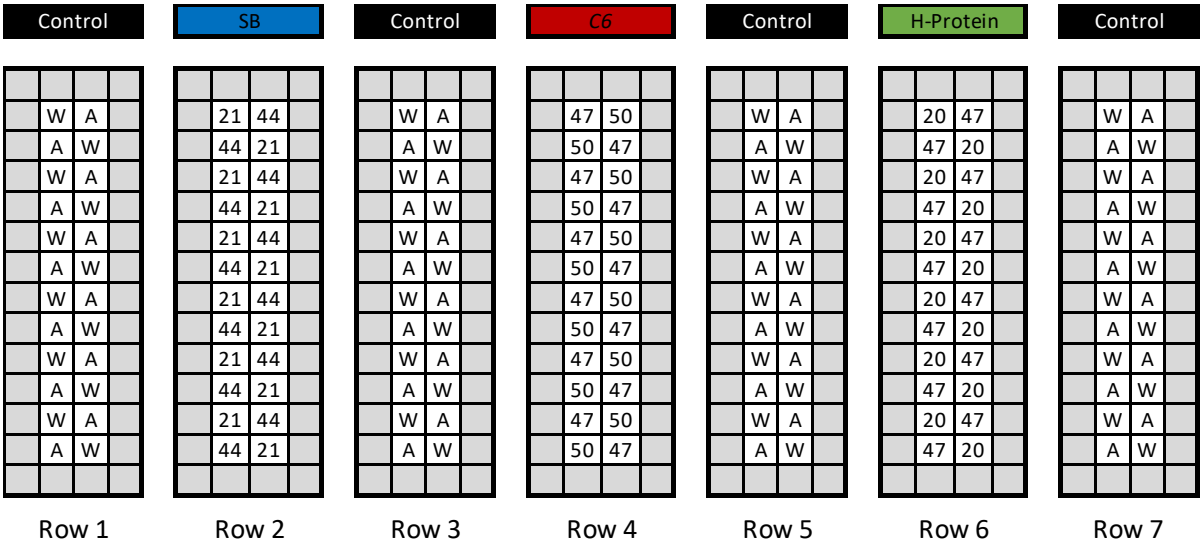


Figure 4.2. Schematic representing the filed experimental design. The field experiment was planted to a replicated control design. Each construct was straddled with its two representative controls. Each row contained two 2x11 experimental plants. The grey boxes represent boarder plants. The whole experiment was also enclosed in two rows of boarder plants. In control row W=wildtype, A=azygous. The trial in this study has 7 individual rows of 4x14 plants, including 2x12 experimental plants and a wildtype border to normalise edge effect. Of the seven rows, rows 1,3,5 and 7 were control blocks and row 2, 4 and 6 were S_B, *C6* and H-protein respectively. Each manipulation used two independent lines of T2 homozygous seed. The control plants were a mixture of wildtype and f azygous plants. Two genotypes were used for each construct, these are represented by the different numbers.

4.3 No differences were observed between the transgenic lines and control plants during the early growth phase in the glasshouse

The early growth of the plants being used for this field trial was measured by determining the leaf area of a subset of plants from each line. Five plants were used per line and the first measurement was taken two days after being moved into individual pots (14 days after sowing) and then repeated for 7 days. Measurements stopped after this time due to leaves beginning to overlap and thereby making the measurements less accurate. The initial measurements showed no significant difference between any of the individual lines, the means being between 0.45 and 0.75 cm^2. The final measurement was conducted 20 days after sowing and there was no difference between the transgenic lines when compared to the control group (CN (both wildtype and azygous)). The transgenic line, *$C_6$47*, showed the greatest difference having a leaf area at 20 days of 3.3 cm^2 compared to the controls of 5.06 cm^2. However, this was not significantly different (P=0.0503) (Fig. 4.3).

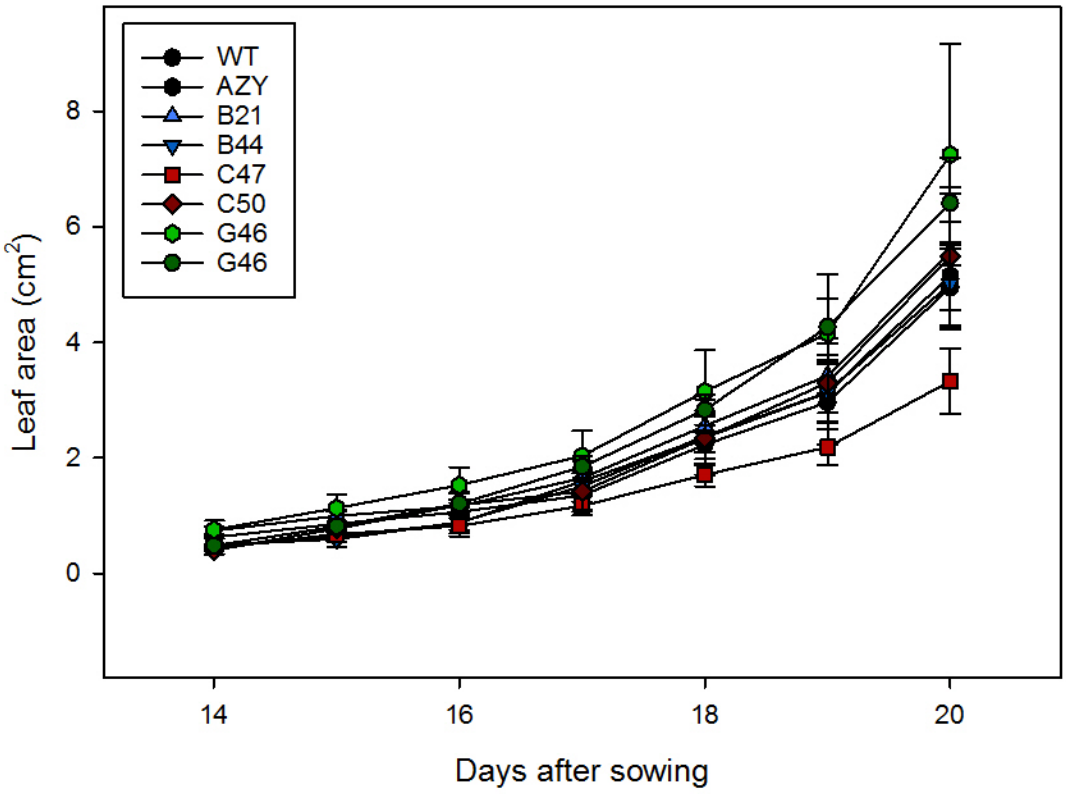


Figure 4.3. Expression of introduced proteins relay no change to the rate of early growth. Plants grown and germinated in glasshouse conditions were measured for leaf surface area from 14-20 days after sowing. Aerial photos were taken from a subset of 5 plants per line and leaf surface area was derived using. After 20 days newly developed leaves would overlap previous leaves making measurements less accurate. Five replicates were used per genotype per day. T-tests were used to determine if any of the transgenic plants differed from the combined control group (wildtype and azygous) on each given day. No significant differences were observed.

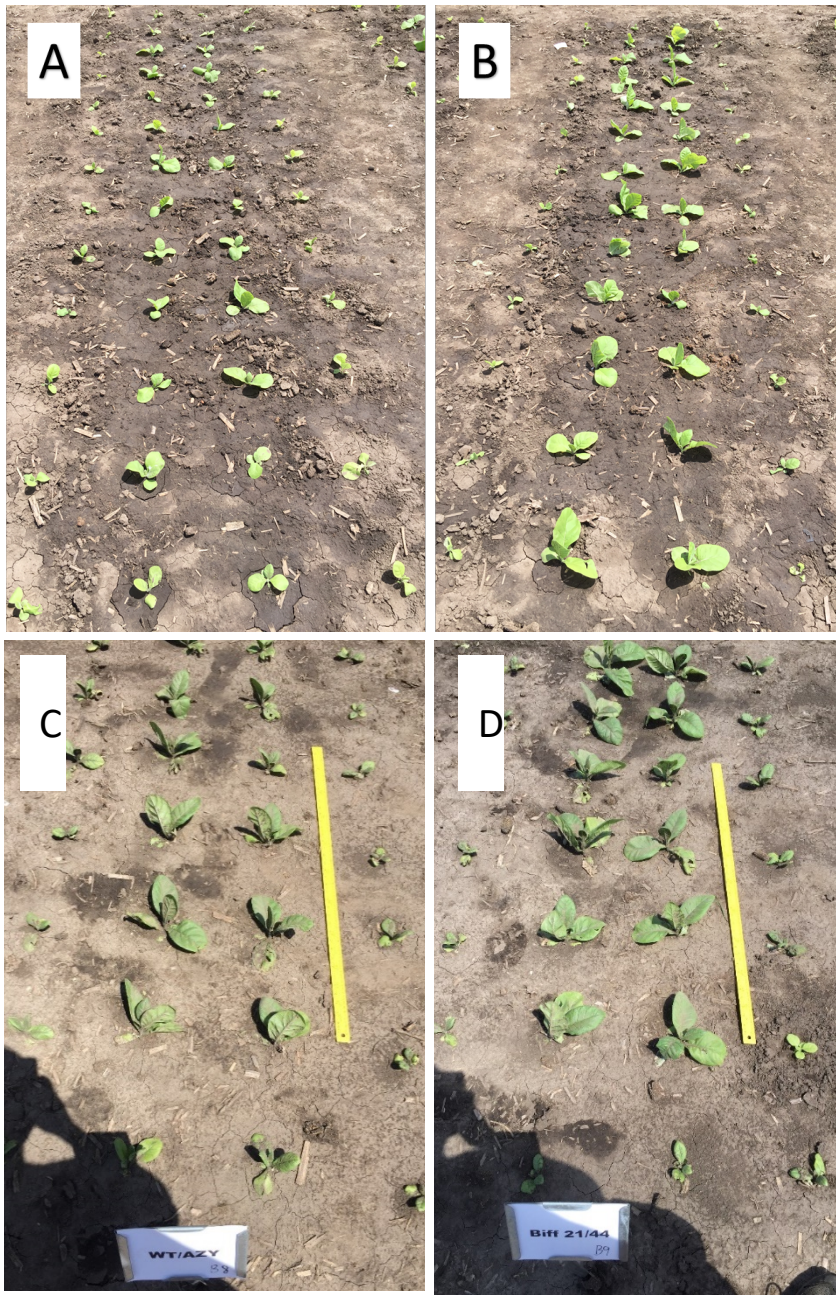


Figure 4.4. Photographs of the transgenic plants in the field showing differences in size. Photographs were taken of row 1 (A, C), a control line for row 2 (B, D) the S_B expressing lines. (A, B) were taken two days after transferring plants the field and (C, D) were taken 10 days after being transplanted in to the field. The yellow ruler was 1 meter in length.

4.4 Overexpression of S_B, *C_6* and H-protein increases above ground plant biomass in field conditions

The main focus of this work was to determine the impact of the individual manipulations on plant biomass. The same harvest point was used in the field trials as in the glasshouse experiments (Lopez-Calcagno et al., Unpublished, Lopez-Calcagno et al., 2019), and plants were harvested once the first bud was visible. In the field trial this occurred 14 days after being transplanted from the glasshouse into the field and 40 days after sowing.

During the harvest measurements were taken of leaf number, stem length and leaf area. All three transgenic manipulations showed significant increases across all measured parameters (Fig. 4.5). The leaves and stems of individual plants were separated out and dried, then, the weights determined to give the above ground biomass. Significant increases in above ground biomass were observed when compared to wildtype. The S_B, *C_6* and H-protein manipulations had increases of 24 %, 38 % and 4 3% respectively.

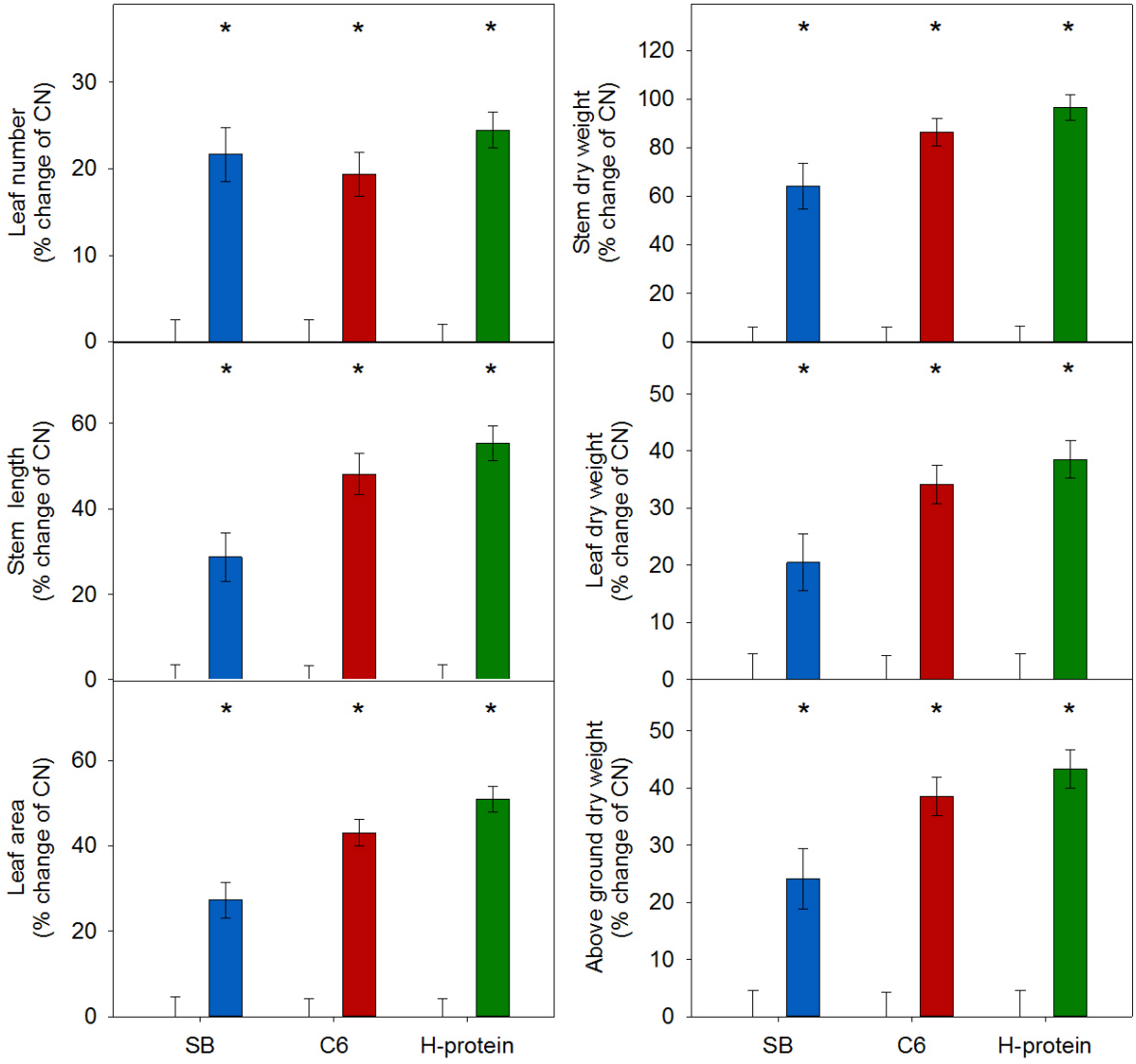


Figure 4.5. Harvest data from field trials shows increases in growth for the transgenic lines. Harvest data from 40-day old plants. Plants were germinated and seedling grew in the greenhouse for 26 days before being transplanted into the field for further 14 days. Leaf number and stem length were measured during harvesting and leaf area was measured on an area meter (LI-3100C Area Meter, LI-COR). Leaf and stems were separated and dried at 60 ^{o}C in a custom built drying over, and once dried weighed. Figures are displayed as percentage of controls (CN), where the control group is the combination of the two adjacent rows to the transgenic group. Error bars represent standard error. Absolute values are stated in table 4.1. 2016 biomass harvest data was tested for homogeneity using the Brown-Forsythe test and normality was determined using Shapiros-Wilks test, which was followed with a T-test. *indicates p values <0.05

Mean values						
Genotype	Leaf Number	Stem Height (mm)	Leaf area (cm^2)	Leaf dry biomass (g)	Stem dry biomass (g)	Above ground biomass (g)
Control (Bifunctional)	6.44	98.79	441.80	3.71	0.34	4.05
B21	7.83	127.17	562.34	4.47	0.56	5.03
B44	7.92	128.08	568.23	4.68	0.55	5.23
Control (Cytochrome c_6)	6.56	101.98	411.40	3.63	0.32	3.95
C2	7.42	134.92	569.63	4.78	0.51	5.29
C3	8.25	167.25	607.53	4.96	0.69	5.65
Control (H-protein)	7.06	101.00	389.45	3.44	0.31	3.76
G20	8.67	169.83	569.67	4.61	0.64	5.25
G46	8.92	144.00	605.91	4.93	0.59	5.52

Standard error						
Genotype	Leaf Number	Stem Height	Leaf area	Leaf dry biomass	Stem dry biomass	Above ground biomass
Control (Bifunctional)	0.16	3.40	20.31	0.17	0.02	0.19
B21	0.41	9.90	39.05	0.30	0.09	0.39
B44	0.29	10.77	28.90	0.33	0.06	0.37
Control (Cytochrome c_6)	0.17	3.25	17.54	0.15	0.02	0.17
C2	0.26	5.55	23.20	0.20	0.04	0.23
C3	0.25	11.78	28.31	0.26	0.04	0.28
Control (H-protein)	0.14	3.61	16.74	0.16	0.02	0.17
G20	0.19	8.29	24.40	0.20	0.04	0.23
G46	0.31	8.48	25.98	0.25	0.05	0.28

Table 4.1. Harvest data means raw values. Harvest data (a) presented as the mean values and (b) with the related standard error. Control groups are the means of the combined rows that relate to the relevant lines.

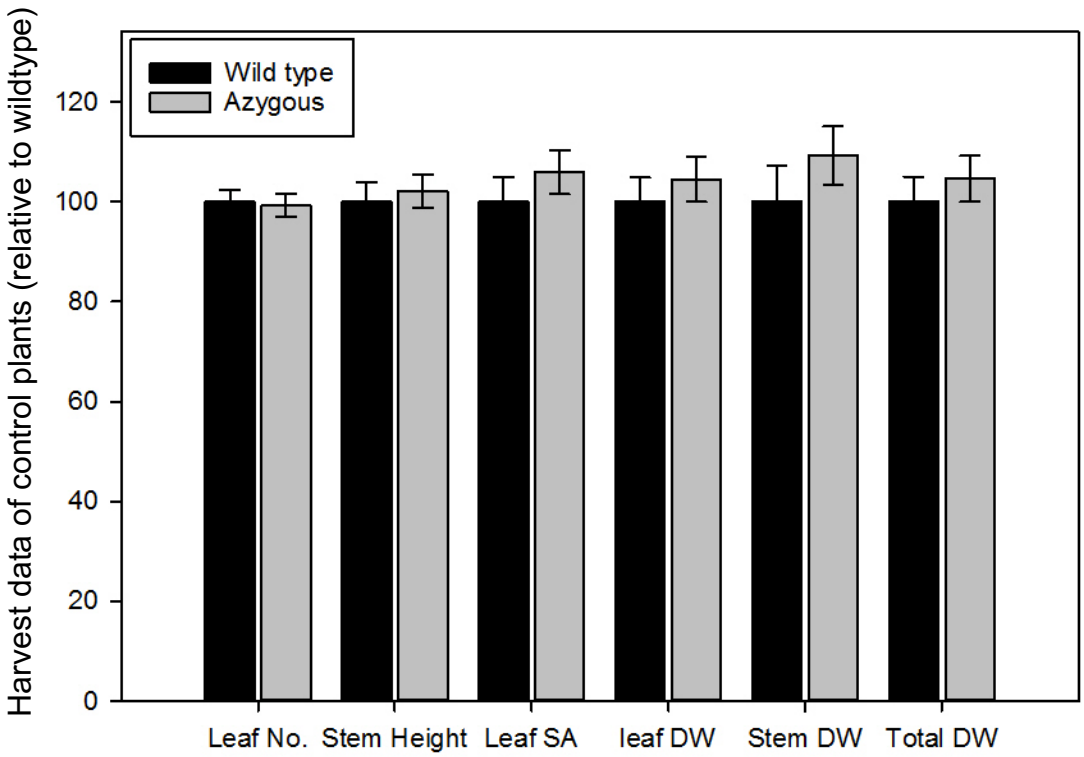


Figure 4.6. Harvest data from both the azygous and wildtype controls. Harvest data from the control lines was separated to gain an understanding on how both sets of controls acted in the field conditions. There were no measurable parameters in which the controls performed significantly differently to each other. Data was grouped as wildtype and azygous plants, T-tests were used to determine differences. No parameters showed differences.

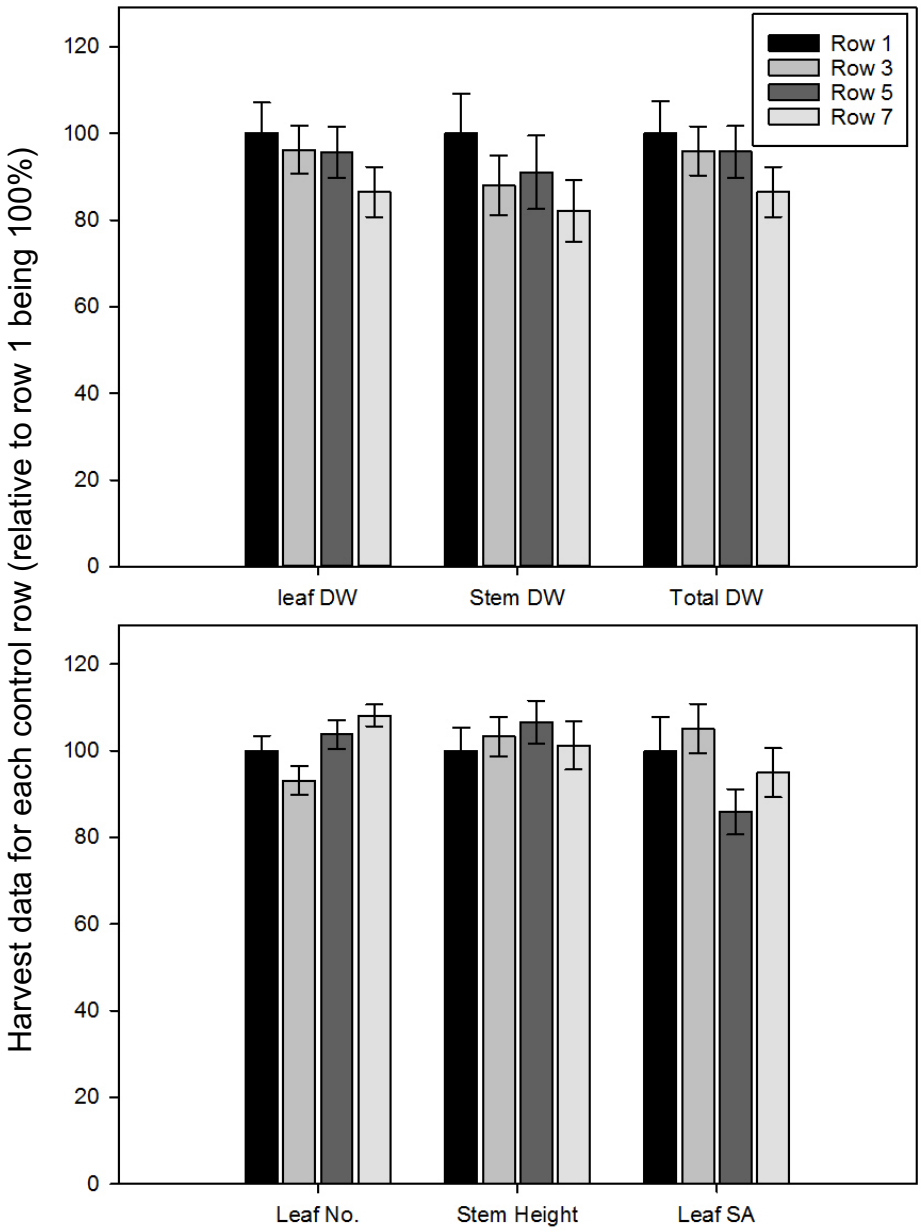


Figure 4.7. A comparison of control harvest data from individual rows. The individual rows of control plants were compared to determine how homogenous the growth was across the field, for the current project the parameter of most importance is the total dry weigh (Total DW) and there was no significant difference between the rows. All the data was presented as a percent change to Row 1. T-tests were used to compare each row to row 1. Refer to figure 4.2 for the field trial layout.

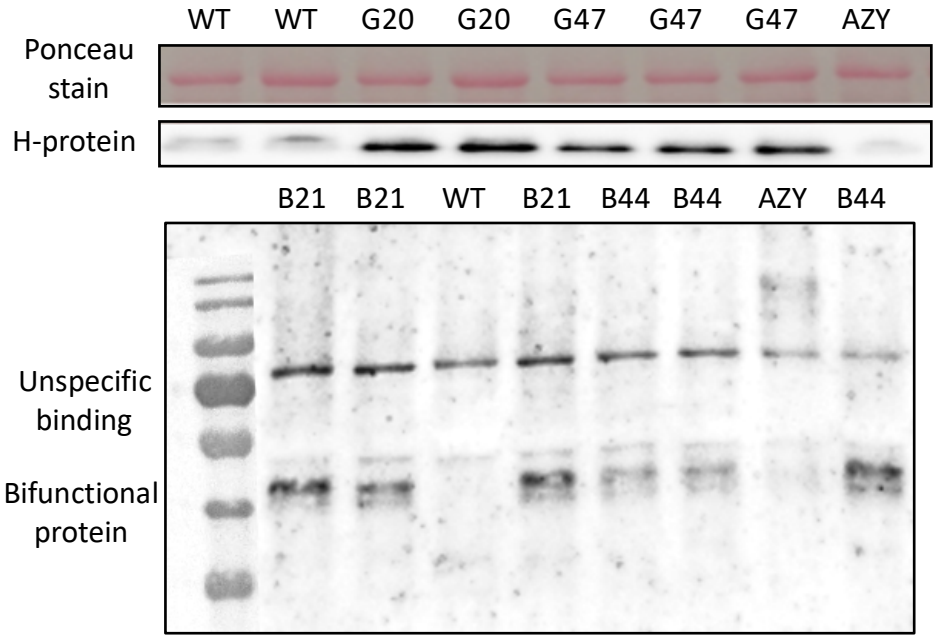


Figure 4.8. Immunoblot analysis from leaf disks using custom raised antibodies. Immunoblot analysis of protein extracts from the newest fully expanded leaf of field grown transgenic and wildtype plants. Approximately 8 µg of protein was separated on a 12 % polyacrylamide gel and blotted onto nylon membrane and probed with polyclonal antibodies against the Bifunctional FBP/SBPase, H-protein (Timm *et al.,* 2012). Ponceau staining was used as a loading control for H-protein. A nitrocellulose membrane with a 0.45 µm pore size was used during the transfer of proteins. B21, B44, G20 and G47 refer to lines used in the experiment (Fig. 4.2)

Discussion

Transgenic tobacco plants were produced to independently express cytochrome c_6, FBPase/SBPase and to overexpress the H-protein of the glycine cleavage system. These plants were grown in field conditions to evaluate their performance. These transgenic plants had previously been grown in greenhouse experiments where they provided positive results in terms of photosynthesis and productivity (Lopez-Calcagno et al., Unpublished, Lopez-Calcagno et al., 2019). Here, two T2 lines for each line were evaluated for their productivity in field trials. The plants were arranged in a replicated control experimental design and showed significant increases in biomass ranging between 124 and 143 % (Fig. 4.5) in comparison to their respective controls.

The 2016 field experiment was based on a 'repeated control design' which is used in to determine yield in trials of new varieties of crops. This is structured to maximise the number of different varieties to be screened in a relatively small area. This experimental design provides the opportunity for comparison between individual treatments and controls. However, the drawback of the repeated control design is the lack of the ability to undertake robust statistical analysis. The design means that T-tests were used to determine whether there are any changes between a transgenic line and the adjacent control lines. Due to the design of the experiment it is not possible to directly compare between the transgenic rows. This is due to the transgenic rows not being grown in replicate, and so not being controlled for the position affect across the field. While this is the case, comparing the different control rows with one another gives an indication to how the position in the field will affect the growth of the individual rows. The comparison of the four independent control rows shows there are no trends and no significant difference between them when comparing the growth phenotype (Fig. 4.7). This indicates that the

microenvironment of the field, in relation to each row, is not having an effect on the growth of the plants. The largest difference in total biomass in the control plants was between row 14 and row 8, with a difference of 12 %, but analysis of these data showed that this was not statistically significant. As there is no detectable positional effect of the control lines, it is possible to compare the transgenic lines with each other.

4.5 Plant growth and development

Each of the individual lines was germinated in glasshouse conditions before being moved into individual pots. The day after being moved a sub set of 5 plants from each line was photographed and the leaf area determined. The means of each line ranged from 0.45 to 0.75 cm^2 with no values being significantly different from the controls. Having no lines significantly different from the controls in the first measurement showed that the differences in rates germination and early growth was biasing the experiment. The leaf area was analysed over the next seven days, and at no point did any of the transgenic lines significantly differ from the control plants. As there was no difference after this time it would indicate that any difference that are observed are not due to advantages conferred due to early germination and establishment of seedlings.

The plants were grown in the glasshouse for 18 days before being transplanted to the field. The harvest data (Fig. 4.5) shows the transgenic plants had significant increases in leaf number, leaf area and stem length. The total above ground dry biomass had also significantly increased for the three transgenic constructs by 24, 38 and 43 % for S_B, *C6* and H-protein respectively. These increases in biomass were representative of the changes that were observed in glasshouse conditions (Lopez-Calcagno et al., Unpublished, Lopez-Calcagno et al., 2019). The dry biomass was measured as stems and leaf's separately, and the largest change in biomass was seen in the stem dry weight,

where the transgenic lines were between 164 and 196 % of the control lines. When growing tobacco plants with a synthetic pathway, replacing photorespiration, South et al. (2019) observed a similar trend with the dry weight of the stems being increase more than the leaves. However, South et al. (2019) harvested at a later date, leaving them in the field for 28 days, opposed to 14 days as we did, although both experiments see the largest difference in biomass in the stem of the plant.

The field trials harvest data was compared with the same seed batch grown in glasshouses at the University of Essex, UK. This showed that although the plants were harvested at the same stage of development, after approximately the same amount of days since sowing, (Lopez-Calcagno et al., Unpublished, Lopez-Calcagno et al., 2019) the plants were not at the same growth stage. *Nicotiana tobaccum cv*. Petit havana gowns approximately 15 leaves on the main stem when the fully developed (Lopez-Calcagno et al., Unpublished, Lopez-Calcagno et al., 2019). Due to plants usually losing the first true leaves before harvest and whether the small leaves around the head of the flowers sometimes are included in the counts, the number of leaves can only be estimated. However, as the plants had between 8.5 and 11 leaves, this indicates that neither the controls nor transgenic lines were fully developed, although flowering. The expected leaf number in the field would be ~12 for a plant that has grown to put on the full amount of leaves. This highlights how the plants grow and develop under field conditions, and how this differs from the growth characteristics of plants grown in glasshouse conditions. Subsequently, in future experiments this difference in growth characteristics will be considered when concluding on the most appropriate time of harvest.

The data presented in this chapter demonstrates the potential of the transgenic lines studied to increase plant biomass and provided the basis for the larger studies undertaken in Chapter 5.

Chapter 5 – Replicated field trials determining how multiple manipulations to the photosynthetic pathways affect the productivity of tobacco plants (2017 and 2018)

Introduction

The previous chapter explored how manipulating single genes involved in photosynthesis (*C6*, S_B and H-protein) could lead to a significant increase in total biomass of *Nicotiana tobacum* cv Petit havana plants. Concurrent experiments conducted in glasshouse conditions, on the same seed batch (Lopez-Calcagno et al., 2019, Lopez-Calcagno et al., Unpublished), have demonstrated the effect that manipulations have on both photosynthetic capacity and growth. The glasshouse trials included plants overexpressing the H-protein, *C6*, S_B and the double cross of *C6* and S_B. The trials demonstrated an increase in biomass of *C6* and S_B expressing plants, which increase was significantly larger for the double manipulation that expressed the *C6* and S_B genes in tandem (Lopez-Calcagno et al., Unpublished). Furthermore, plants overexpressing H-protein were also tested and the phenotype was equally promising (Lopez-Calcagno et al., 2019). These glasshouse studies, combined with the field trials from the 2016 season, provided a substantial amount of evidence to the value of undertaking full scale field trials to full phenotype the growth patterns of the transgenic plants. For the study to explore whether multiple manipulations to the photosynthetic pathways can provide an additive effect to productivity, the experiment uses single, double and triple manipulations.

This previous field trial (chapter 4), held in 2016, followed the harvest protocol that is used in glasshouse experiments, which is to harvest as the first bud forms. The reasoning for this approach is to harvest the plants before senescence starts to occur. However, when tobacco plants are grown in field conditions, flowers begin to appear earlier in development as opposed to glasshouse grown plants. The biomass of plants harvested from glasshouse trails and field trials is extremely different. When CN plants are harvested after 40 days, the plants grown in glasshouse conditions reached a biomass

of 9 g, whereas the same seed batch grown in field trails for the same length of time, reached a final biomass of 3.75 g (Lopez-Calcagno et al., 2019). Given the difference in growth phenotype, it was identified a clear need to implement an approach determining the correct harvest time. Otherwise, the timespan would not be sufficient for collecting data in relation the photosynthetic capacities of the plants. Whilst looking for differences in phenotype caused by manipulation to NPQ, Kromdijk et al. (2016) conducted gas exchange analysis after the buds had formed and harvested once the first flower opened. The work by Kromdijk et al. (2016) and the 2016 field trials, indicate that the stringent timeframe used in glasshouse trials is not feasible in field trials.

The designs used in the field vary depending on the hypothesis, and there is a variety of trial designs published that have used tobacco. The design used in the previous chapter was a ‘repeated controls’ design, explained in Lopez-Calcagno et al. (2019). This design allows several genotypes to be grown in a relatively small area but lacks some statistical power. Repeated single genotype blocks were used by South et al. (2019), while complete block design was utilised by Kromdijk et al. (2016), which are both virtuous methodologies and would have been appropriate for fully replicated tobacco trials. An alternative option for trailing several genotypes is the incomplete blocks (Lopez-Calcagno et al., 2019). This methodology uses blocks with one genotype per manipulation, whereas several blocks combined will make a complete set of genotypes.

The experimental design used in 2016 provides a large abundance of data whilst using a limited amount of resources. Changing the methodology could provide opportunities that enrich the collected data and provide a more statically robust result. This was the case in the 2016 trials, due to the lack of transgenic replication in the design. As a result, an

incomplete block design was utilised, as described by Lopez-Calcagno et al. (2019), for both of the 2017 experiments.

Designing an experiment that uses suitable control plants is important to ensure validity. Field trials using tobacco have used a wide range of control plants, some experiments only use wildtype plants (Kromdijk et al., 2016, Lefebvre et al., 2005, Rosenthal et al., 2011). The limitation to only using wildtype plants is the plant not having been through tissue culture. Plants that are transformed through tissue culture are subject to somaclonal variation, which is the defined as the genetic variation observed amongst progeny of plants regenerated in tissue culture from a single somatic cell. The cause of this variation is down to gene amplification or chromosome shuffling that occurs during the tissue culture process (Morrison et al., 1988). For this reason, a more suitable control would have been subjected to the growth conditions that occur during the tissue culture process. South et al. (2019) used both wildtype plants and empty vector transgenic plants. The empty vector plant is transformed with the entry vector, containing the selection marker gene, but without the gene of interest. Yet, the plants are recovered in the same fashion that other transgenic plants would be. An alternative to using an empty vector is to use an azygous, null sergeant plant (Lopez-Calcagno et al., 2019). Once a plant has been through the transformation process, the genome is unstable and will be segregating. The azygous plants are segregates that have lost the gene of interest. The most suitable control is a matter of opinion, but it is important to include plants that have been through the tissue culture process to confirm that any observed differences in the growth of transgenic plants are caused by the inserting the gene of interest and not an artefact of somaclonal variation.

The previous chapter trailed plants that had manipulations to genes expressing the bifunctional FBPase/SBPase protein (S_B), the cytochrome *c_6* protein (*C_6*) and the H-protein of the glycine cleavage system. The same transgenic lines were crossed to produce a double-expressing plant that had both the S_B and C_6 (D) genes, and a triple-expressing line consisting of S_B, *C_6* and H-protein (T) (Fig. 5.1).

The aim of this chapter is to measure the effect of expressing S_B, *C_6* and the H-protein as single manipulations. In addition to the single gene manipulations, the chapter identifies whether the expression of S_B and *C_6* as a double manipulation, and S_B, *C_6* and the H-protein as a triple manipulation has an accumulative effect on the productivity of *N.tobacum.*

Results

5.1 The experimental design for the field trials

Two field trials were held in 2017; both experiments had the same design of incomplete blocks, within complete rows, as described by Lopez-Calcagno et al. (2019) (Fig. 5.1), and included three independent genotypes per manipulation. Experiment 1 used three lines of S_B (S_B3, S_B6 and S_B21), three lines of H-protein (G04, G20 and G46) and three lines of C_6 (C41 C47 and C50). The control group (CN) contained three lines of azygous control, segregated from the plants generated during transformation and seeds from three wildtype plants that had been grown alongside the previous generations of plants. Seeds were left to germinate for 12 days before being moved to the hydroponic tray where the seedlings were left to grow for a further 24 days before being transplanted into the field and grown for another 30 days before they were harvested.

Experiment 2 used the same control group comprising of azygous and wildtype plants. Three lines of C_6 (C15, C47 and C50), three lines of the double-crossed manipulation (D1, D2 and D6) and three genotypes of the triple manipulation (T1, T2 and T3) were used. The individual genotypes from the crossed plants (D and T) were plants that had a different parental background, i.e. D genotypes were crossed from several S_B and several C_6 plants. The seedlings were germinated under the same conditions as in experiment 1. The seeds were left to germinate for 12 days before being moved into the hydroponic trays, where they grew for 20 days before being transplanted into the field. After 29 days of further growth, the plants were harvested.

The 2018 experiment had a similar design to the 2017 experiments but was larger and included four manipulations of which each had three independent lines. The genotypes used were: S_B (S_B6, S_B21 and S_B44), C_6 (C15, C47 and C50), the double S_B crossed with

C_6 (D2, D3 and D6), and the triple manipulation (T1, T4 and T5). As described for the 2017 experiment, two controls were used for this experiment, the wildtype and azygous plants. Both the wildtype and azygous lines were made up from a pool of seed from at least three individual lines. The 2018 experiment, on the other hand, used a complete block design (Fig. 5.2).

The 2018 experiment was designed to include five individual harvest points; the first shortly after the seedling plants were moved in to the hydroponic trays (19 days after sowing), the second was the day the plants were transplanted into the field (28 days after sowing), where a representative subsection was kept separate for harvested. The next three were at different stages during growth in the field. Although this provides interesting data, only the last harvest point will be used in this discussion. The reasons for this, is due to this harvest point being taken at the same developmental point as both 2017 experiments, and it is the closest in terms of days after sowing. Subsequently, this harvest is the closest comparable point to both the other experiments.

All of the plants grown, in both 2017 and 2018, were analysed via iDna Genitics ltd to determine whether the genotype was stable, this was done on plants grown in the glasshouse from the same seed batch. All single manipulations (S_B, *C_6* and H-protein) were either T2 or T3 homozygous and the crosses (D and T) were F3 homozygous. The only exception to this was 'Triple 3 (T3)' from experiment 2 in 2017 that was a stable F1 hemizygous. In each block four plants were used for each repetition.

5.2 Generation and selection of transgenic lines

The plants used in the previous chapter form the basis of the work conducted over the 2017 and 2018 field seasons. The constructs (Fig. 5.2A) used were overexpressing for H-protein and expression *C_6* and the S_B genes, the generation of these constructs are

described in previous studies (Lopez-Calcagno et al., 2019, Lopez-Calcagno et al., Unpublished). The crosses were made as described in chapter 2.6. The double cross was progeny of C_6 and the S_B expressing plants. The triple cross has the H-protein crossed into stable double lines. Each genotype was grown in glasshouse conditions for a preliminary growth phenotype and for molecular characterisation (Lopez-Calcagno et al., 2019) to confirm the presents of the genes of interest. The first generation after the cross (F1 generation) will have a non-segregating hemizygous genotype. The F2 generation will segregate and it is at this stage that a homozygous plant can be selected. The seeds of this will be a genetically stable F3 homozygous plant. All of the crosses that were put into the field were of the F3 generation, except for the T3 genotype for experiment 2 of 2017. T3 was F1 hemizygous; qPCR analysis had demonstrated how this plant was transcribing the RNA on a comparable level to the F3 homozygous genotypes. The single gene manipulations used in these experiments were T2 homozygous.

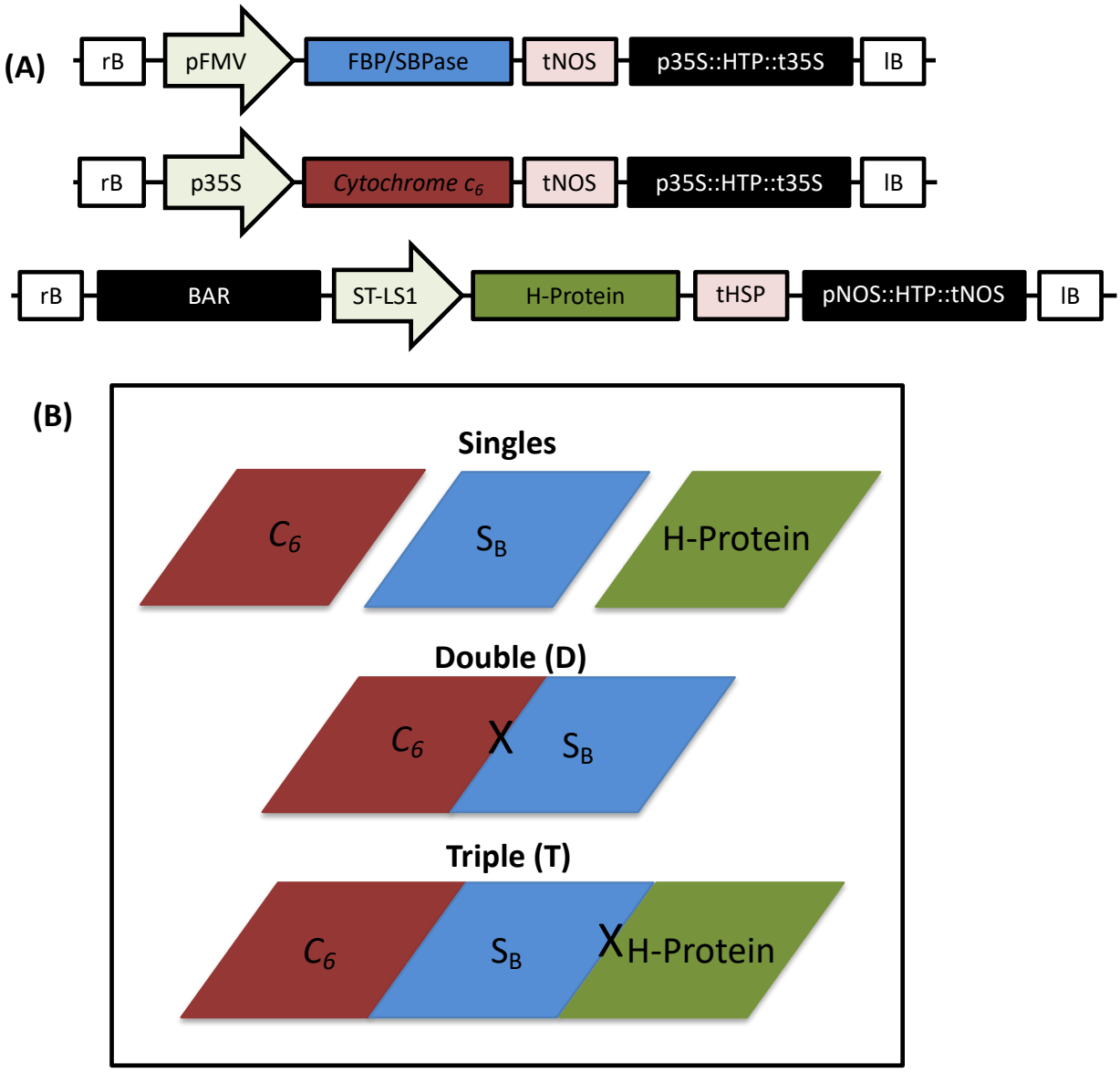
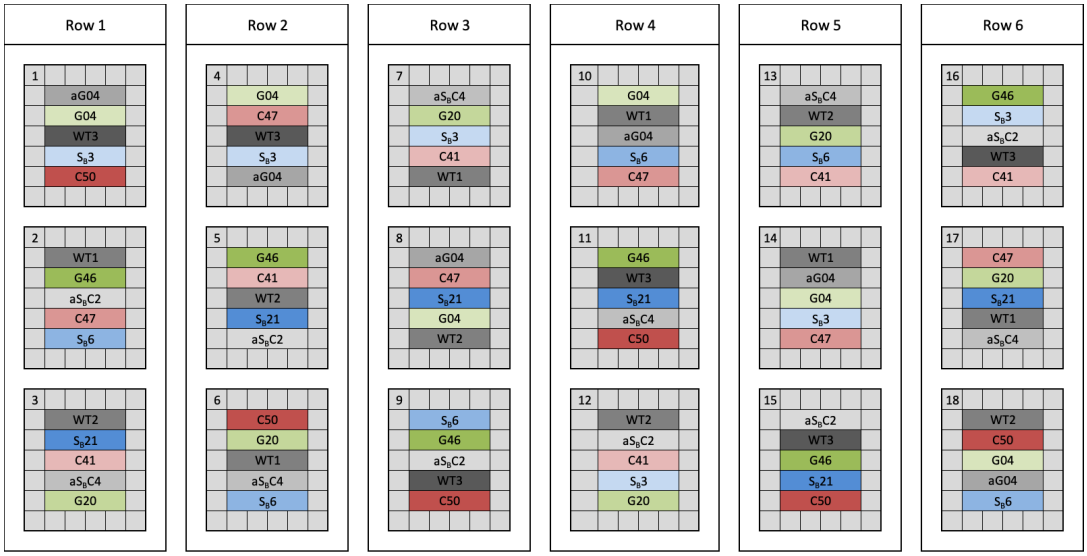
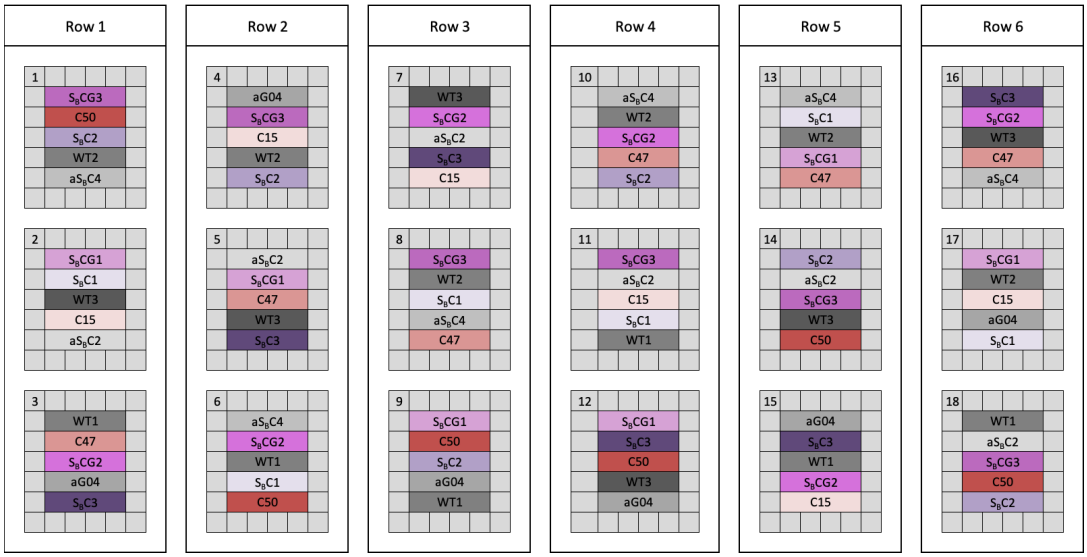


Figure 5.1. Schematic representation of the constructs used for the transgenic plants, and how the crosses were assembled. (A) FBP/SBPase, cytochrome c_6 and H-Protein constructs for expression in *N. tobacum* cv. Petit Havana. **(B)** representation of the manipulations used in the experiments, three single gene manipulations. One double manipulation crossed from multiple C_6 and S_B lines (D). And one triple manipulation, crossed from D and H-Protein plants (T).

(A)


Row 1
1
aG04
G04
WT3
SB3
C50
2
WT1
G46
aSBC2
C47
SB6
3
WT2
SB21
C41
aSBC4
G20
Row 2
4
G04
C47
WT3
SB3
aG04
5
G46
C41
WT2
SB21
aSBC2
6
C50
G20
WT1
aSBC4
SB6
Row 3
7
aSBC4
G20
SB3
C41
WT1
8
aG04
C47
SB21
G04
WT2
9
SB6
G46
aSBC2
WT3
C50
Row 4
10
G04
WT1
aG04
SB6
C47
11
G46
WT3
SB21
aSBC4
C50
12
WT2
aSBC2
C41
SB3
G20
Row 5
13
aSBC4
WT2
G20
SB6
C41
14
WT1
aG04
G04
SB3
C47
15
aSBC2
WT3
G46
SB21
C50
Row 6
16
G46
SB3
aSBC2
WT3
C41
17
C47
G20
SB21
WT1
aSBC4
18
WT2
C50
G04
aG04
SB6

(B)


Row 1
1
SBCG3
C50
SBC2
WT2
aSBC4
2
SBCG1
SBC1
WT3
C15
aSBC2
3
WT1
C47
SBCG2
aG04
SBC3
Row 2
4
aG04
SBCG3
C15
WT2
SBC2
5
aSBC2
SBCG1
C47
WT3
SBC3
6
aSBC4
SBCG2
WT1
SBC1
C50
Row 3
7
WT3
SBCG2
aSBC2
SBC3
C15
8
SBCG3
WT2
SBC1
aSBC4
C47
9
SBCG1
C50
SBC2
aG04
WT1
Row 4
10
aSBC4
WT2
SBCG2
C47
SBC2
11
SBCG3
aSBC2
C15
SBC1
WT1
12
SBCG1
SBC3
C50
WT3
aG04
Row 5
13
aSBC4
SBC1
WT2
SBCG1
C47
14
SBC2
aSBC2
SBCG3
WT3
C50
15
aG04
SBC3
WT1
SBCG2
C15
Row 6
16
SBC3
SBCG2
WT3
C47
aSBC4
17
SBCG1
WT2
C15
aG04
SBC1
18
WT1
aSBC2
SBCG3
C50
SBC2

(C)

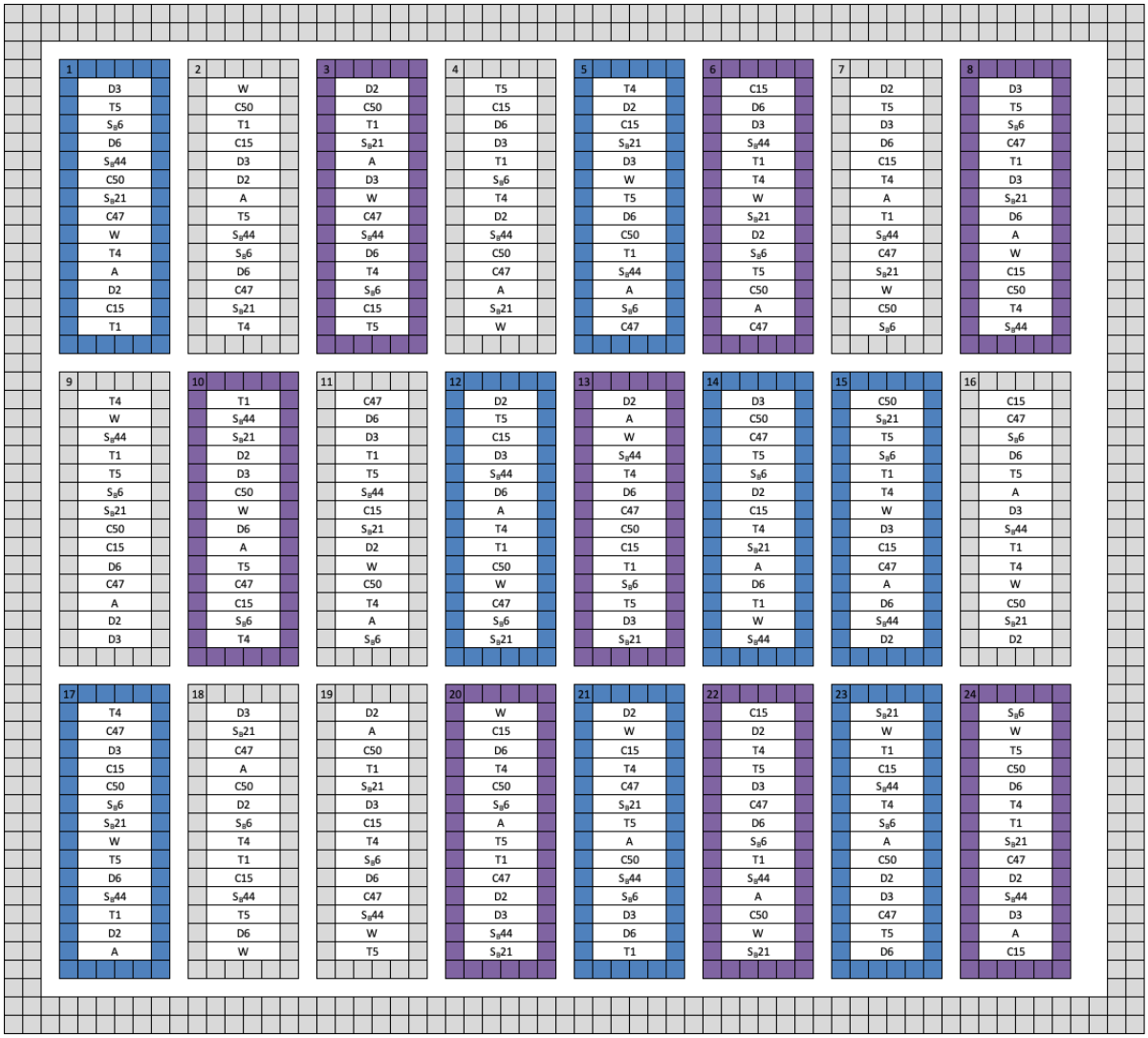


Figure 5.2. Field experiments layouts. Schematics to represent the experimental field designs for both 2017 experiments and the 2018 trial. (**A, B**) Both 2017 field design were incomplete block, complete row design. The randomised rows split into three blocks, where one row has all 15 lines and one block had one line of each construct. Rows were randomised using RAND function, Microsoft Excel 2010. **(C)** complete block design with. The colour outlining each block represents the three harvest, the purple outline was harvest 1, grey outline was harvest 2 and the blue outline was harvest 3. The genotypes were randomly assigned to the blocks. The blocks were designated to each harvest point with a random number generator. All three experiments surrounded had a wildtype border around each block and each separate experiment was bordered by two lines of wildtype. All transgenic plants had a stable genotype, original transformations were T2 homozygous and crossed transgenic plants were F3 homozygous, with the exception T5 in the 2018 trial which was F1 hemizygous.

(a)

Mean values						
Genotype	Leaf Number	Stem Height (mm)	Leaf area (cm^2)	Leaf dry biomass (g)	Stem dry biomass (g)	Above ground biomass (g)
Control	11.78	33.94	1087.12	5.31	1.90	7.22
B03	12.17	35.92	1116.58	5.52	2.00	7.52
B06	12.08	33.22	1042.58	4.82	1.78	6.43
B21	12.61	40.85	1307.83	6.45	2.73	9.19
C41	11.92	37.75	960.63	4.83	2.31	7.14
C47	11.35	33.40	1024.94	5.09	1.74	6.83
C50	11.97	35.03	939.43	4.81	1.78	6.59
G04	12.36	36.34	1221.14	5.97	2.26	8.23
G20	11.96	30.26	914.46	4.36	1.61	5.97
G46	12.75	35.55	1204.67	6.09	2.10	8.19

(b)

Standard error						
Genotype	Leaf Number	Stem Height	Leaf area	Leaf dry biomass	Stem dry biomass	Above ground biomass
Control	0.110	0.754	24.206	0.111	0.072	0.172
B03	0.177	1.678	72.599	0.344	0.189	0.518
B06	0.295	1.469	58.840	0.222	0.155	0.429
B21	0.228	1.567	46.249	0.186	0.188	0.344
C41	0.208	1.780	58.890	0.234	0.253	0.461
C47	0.520	1.751	54.582	0.217	0.136	0.337
C50	0.282	1.754	31.515	0.169	0.129	0.264
G04	0.195	1.994	48.984	0.246	0.157	0.377
G20	0.222	1.730	54.449	0.204	0.135	0.320
G46	0.173	1.173	51.797	0.271	0.162	0.394

(c)

Mean values						
Genotype	Leaf Number	Stem Height (mm)	Leaf area (cm^2)	Leaf dry biomass (g)	Stem dry biomass (g)	Above ground biomass (g)
Control	13.02	49.59	1739.83	8.84	3.16	11.99
C15	13.25	51.04	1849.25	9.13	3.43	12.57
C47	12.83	49.50	1803.75	8.62	3.03	11.71
C50	13.25	51.79	1710.54	8.81	3.47	12.28
D1	13.75	52.92	2004.79	10.44	3.82	14.26
D2	12.88	46.83	1648.88	8.63	3.06	11.68
D6	13.38	60.08	2074.75	11.18	4.98	16.16
T1	13.92	53.25	2064.29	10.45	3.85	14.31
T2	14.17	57.25	2260.21	11.22	4.39	15.62
T3	13.46	51.04	1673.08	8.93	3.64	12.57

(d)

Standard error						
Genotype	Leaf Number	Stem Height	Leaf area	Leaf dry biomass	Stem dry biomass	Above ground biomass
Control	0.13	1.80	78.52	0.34	0.20	0.53
C15	0.16	1.65	61.60	0.30	0.18	0.47
C47	0.25	1.99	93.10	0.34	0.23	0.55
C50	0.21	2.02	104.19	0.36	0.27	0.62
BC1	0.20	1.68	69.47	0.41	0.22	0.61
BC2	0.15	2.52	97.64	0.34	0.34	0.66
BC6	0.18	1.54	74.15	0.34	0.36	0.59
T1	0.18	1.70	87.03	0.34	0.23	0.55
T2	0.16	2.37	121.56	0.45	0.30	0.71
T3	0.13	2.13	81.95	0.51	0.32	0.69

(e)

Mean values						
Genotype	Leaf Number	Stem Height (mm)	Leaf area (cm2)	Leaf dry biomass (g)	Stem dry biomass (g)	Above ground biomass (g)
Control	12.81	906.09	3505.67	16.11	13.19	29.30
B21	13.34	900.63	3688.86	17.56	16.35	33.90
B44	13.69	906.28	3717.84	16.98	14.61	31.60
B6	13.19	866.72	2947.19	13.66	11.74	24.91
C15	13.97	898.39	3297.34	16.25	13.39	29.64
C47	12.88	866.41	3207.72	14.87	12.86	27.72
C50	13.16	889.06	3125.81	16.64	12.75	29.39
D2	13.34	852.81	3443.84	17.38	13.71	31.09
D3	13.87	867.50	3461.72	16.58	13.55	30.14
D6	13.63	940.50	3686.32	19.40	15.32	33.52
T1	13.09	919.84	3708.97	19.52	15.74	35.26
T4	13.31	872.58	3512.38	16.72	13.97	30.70
T5	13.71	823.23	2853.13	13.15	11.88	25.04

(f)

Standard error						
Genotype	Leaf Number	Stem Height	Leaf area	Leaf dry biomass	Stem dry biomass	Above ground biomass
Control	0.18	13.01	109.01	0.61	0.46	0.96
B21	0.28	15.94	147.08	0.98	0.78	1.65
B44	0.37	18.47	158.20	0.84	0.84	1.57
B6	0.21	16.20	121.04	0.69	0.61	1.23
C15	0.38	13.08	106.77	0.73	0.66	1.31
C47	0.22	20.65	150.50	0.84	0.82	1.58
C50	0.22	15.71	126.52	0.93	0.69	1.45
D2	0.41	28.22	192.87	1.12	0.87	1.82
D3	0.32	25.26	197.55	1.11	0.92	1.96
D6	0.22	13.24	120.78	2.18	0.84	2.65
T1	0.21	11.78	142.24	1.11	0.76	1.63
T4	0.20	15.49	158.75	0.71	0.67	1.30
T5	0.28	20.19	161.20	0.77	1.02	1.76

Table 5.1. Mean harvest data. Harvest data (a, c, d) presented as the mean values and (b, d, f) with the related standard error. Control groups are the means of the combined rows that relate to the relevant lines.

(A)

(B)

Figure 5.3. photographs demonstrating the experiments. (A) An Arial view of the experiment in the summer of 2017. **(B)** An example of transgenic plants before harvest, plants have a fully closed canopy and are harvested before the first flower produces seed.

5.3 Harvest data from three growing experiments demonstrates an increase in productivity for plants expressing genes in combination

The plants were harvested after the canopy had fully closed (Fig. 5.3B) and approximately 50% of plants had flowered. At the time of harvest, measurements were taken for stem length (plant height) and leaf area, before they were dried and the dry weight of stem and leaf biomass was determined. The data presented in Figure 5.5 shows that several lines had year on year increases over the CN group, in particular S_B21, D6 and T1. There is a consistent trend of plants having higher biomass from the D, T and S_B manipulations (Fig. 5.4). In the 2017 experiments differences were evident in individual lines D1, D6, T1 and T2 (Fig. 5.5), but when the individual lines for each manipulation were grouped, no differences were evident between the D and T manipulations (Fig. 5.4). In the 2018 trial, only T1 showed a statically significant increase in biomass over the CN group. When the genotypes were grouped by manipulation, there was no significant difference between these and CN. The results from the 2017 field trials demonstrated a clear increase in biomass with the double and triple manipulations. However, in the 2018 experiments only an individual line (T1) from the triple manipulation showed any significant change over the CN group (Fig. 5.5). In both years only a single line of S_B had an increase over the controls and none of the C_6 plants grew to have a larger biomass. The H-protein was grown in experiment 1 of 2017 and two of the three genotypes grew bigger than the controls. The total dry weight of the CN group was extremely different for each experiment. The biomass of the CN group for the individual experiments is: Experiment 1 of 2017 ~7.5 g. Experiment 2 of 2017: ~12 g. 2018 experiment: ~29 g (Fig. 5.6).

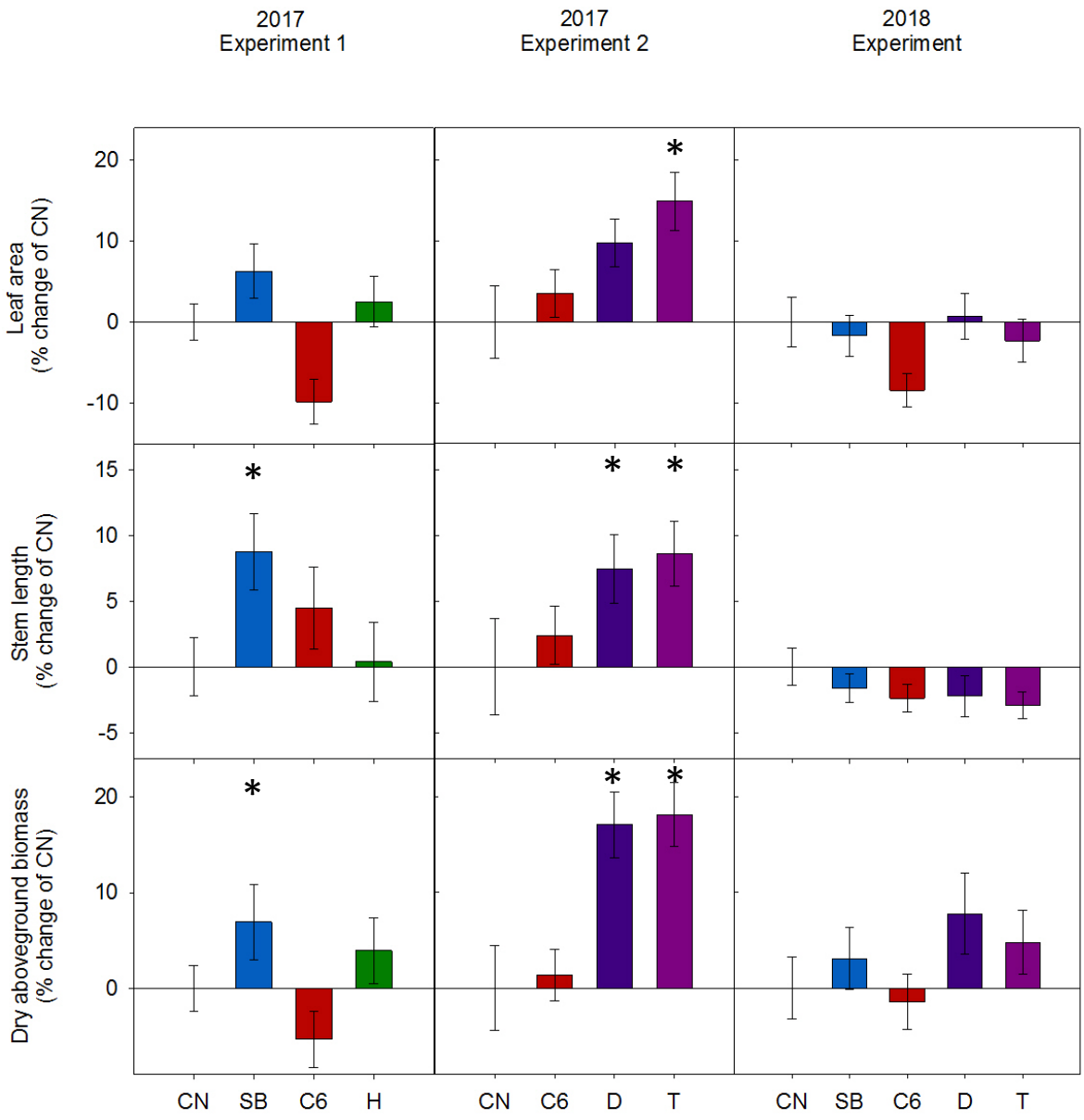


Figure 5.4. Expression of proteins led to a varied growth phenotype throughout the three experiments. Tobacco plants were germinated in growth cabinets and moved to the glasshouse at 10-14 d post-germination, where seedling was transplanted into hydroponic trays. The plants were transplanted into the field trials ~20 days after germination. The plants were harvested once the canopy was closed and over 50% of the plants were flowering. Plants were harvested and plant height, leaf area and above-ground dry biomass (TDW) were determined. The CN group represent both WT and azygous plants. Percent change from CN and SE presented. Linear mix model analysis using lmer and type III ANOVA were used for the analysis. * indicates a difference from controls with a P value of 0.05 or lower.

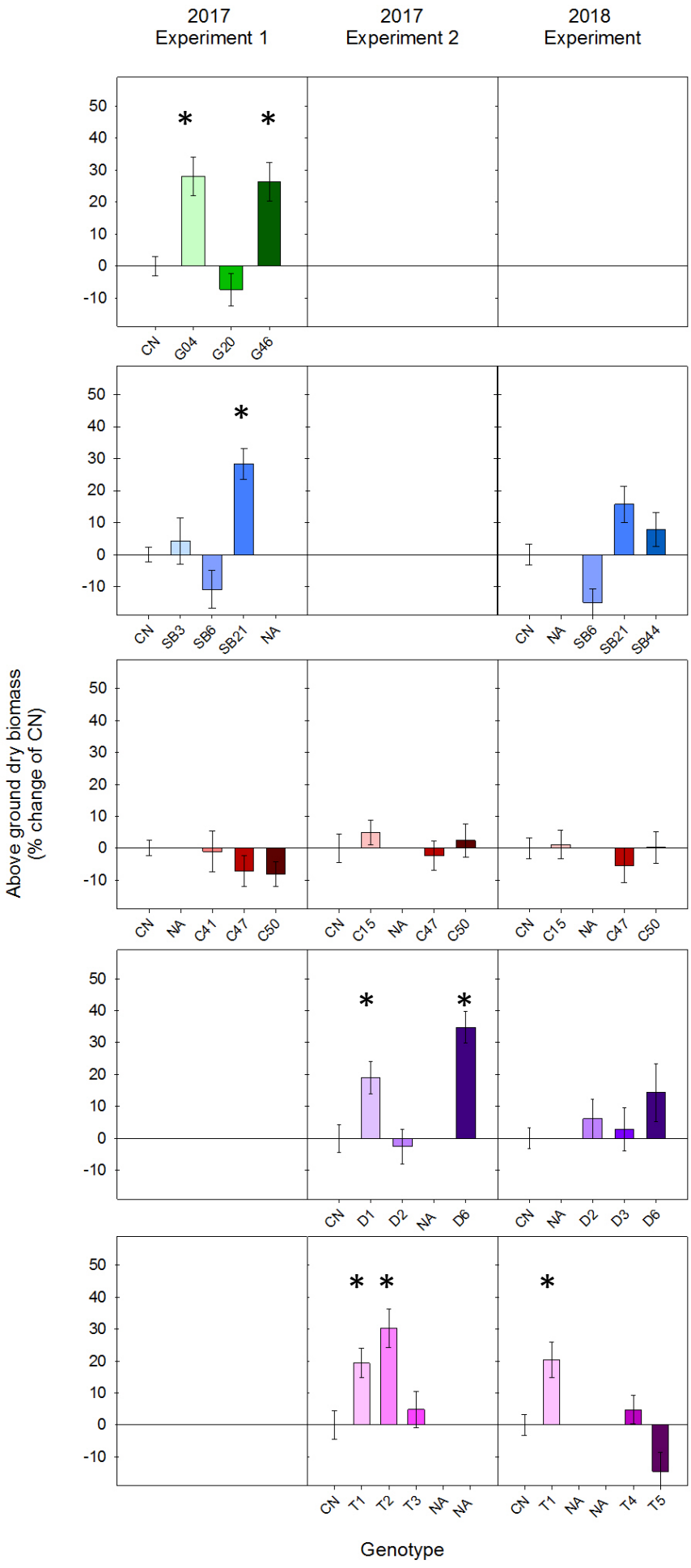

2017
Experiment 1
2017
Experiment 2
2018
Experiment
Above ground dry biomass
(% change of CN)
50
40
30
20
10
0
-10
*
CN
G04
G20
G46
SB3
SB6
SB21
NA
SB44
C41
C47
C50
C15
D1
D2
D3
D6
T1
T2
T3
T4
T5
Genotype

Figure 5.5. Transgenic plants produced a varied growth phenotype between individual lines throughout the three experiments. Tobacco plants were germinated in growth cabinets and moved to the glasshouse at 10-14 d post-germination, where seedling were transplanted into hydroponic trays. The plants were transplanted into the field trials ~20 days after germination. The plants were harvested once the canopy was closed and over 50 % of the plants were flowering. Plants were harvested and plant height, leaf area and above-ground dry biomass (TDW) were determined. Control group represent both WT and azygous plants. Mean and SE presented. Linear mix model analysis using lmer and type III ANOVA were used for the analysis. * indicates a difference from controls with a P value of 0.05 or lower. Error bars indicate standard error and bars labelled NA are place holders for genotypes not used in the experiment.

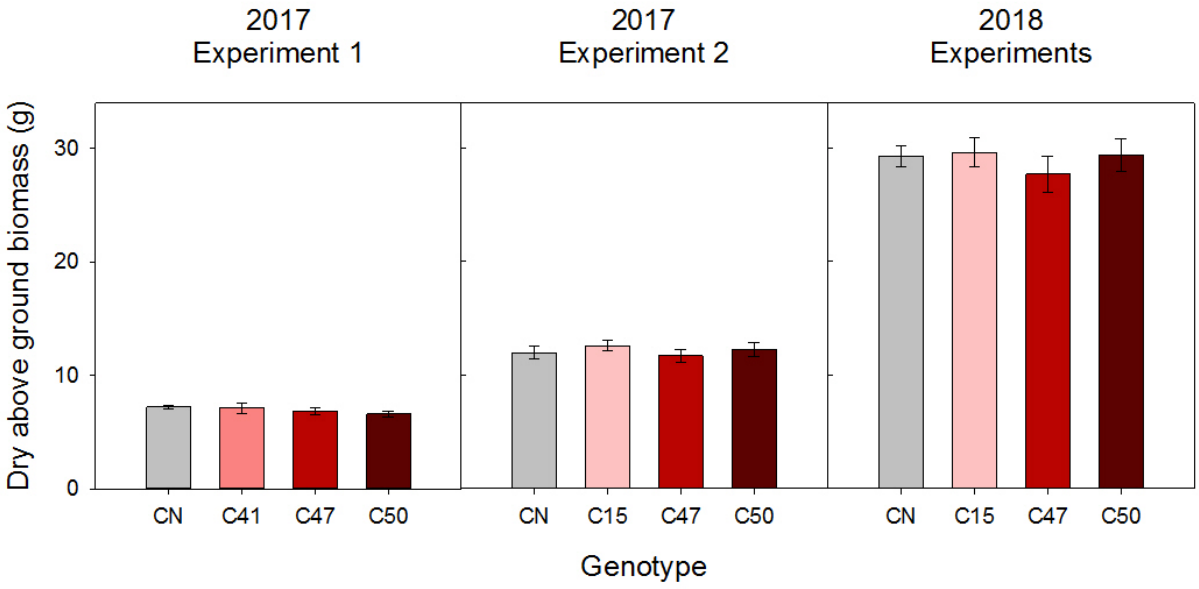


Figure 5.6. Expression of C_6 does not affect the final biomass of plants in three experiments. Tobacco plants were germinated in growth cabinets and moved to the glasshouse at 10-14 d post-germination, where seedling was transplanted into hydroponic trays. The plants were transplanted into the field trials ~20 days after germination. The plants were harvested once the canopy was closed and over 50 % of the plants were flowering. Plants were harvested and plant height, leaf area and above-ground dry biomass (TDW) were determined. Linear mix model analysis using lmer and type III ANOVA were used for the analysis. * indicates a difference from controls with a P value of 0.05 or lower. Error bars represent standard error.

5.4 An increase in carbon assimilation and in intrinsic water use efficiency (*iWUE)* is evident

Previous studies examining these plants under glasshouse conditions have determined an increase in carbon assimilation (Lopez-Calcagno et al., 2019, Lopez-Calcagno et al., Unpublished), but the conditions that plants are grown under and are acclimatised to can affect the physiological parameters. To assess the changes in photosynthetic assimilation of these plants, instantaneous gas exchange measurements were taken. This involved two types of measurements; *A/Ci* and light response curves. The gas exchange measurements were made in the youngest fully developed leaf and took place between 5 and 7 weeks after sowing.

The response of carbon assimilation to an increasing light level (*A/q* curve) was measured at a reference CO_2 of 400 µmol mol^{-1}. For experiment 1 in the 2017 field trial there was no significant difference in the carbon assimilation rates, g_s or *iWUE* between the CN group and the transgenic plants. This lack of difference was seen in experiment 2 of 2017 as well, where there is no significant difference between the transgenic plants and the CN plants. In the 2018 experiments there was an increase seen in carbon assimilation in both the S_B and the D manipulations. The difference in S_B can be observed at light intensities >450 µmol m^{-2}. For the D manipulations the increase in carbon assimilation was seen in light levels >1100 µmol m^{-2}. No difference was seen in *gs* or *iWUE.*

Carbon assimilation was measured as a factor of internal increasing carbon concentration (*A/Ci*), at saturating light levels. The data obtained from these measurements were modelled (Sharkey, 2016) to determine the parameter for *J* and Vc_{max}. The definition of *J* is the maximum rate of electron transport and Vc_{max} is the maximum rate of rubisco carboxylase activity. The results of the models normalise the temperature to 25 ^oC. In experiment 1 of 2017 no difference could be seen in *J,* but the C and S_B plants had a

significant increase in Vc_{max}. In both experiment 2 of 2017 and the 2018 experiment, no difference in Vc_{max} or J were observed.

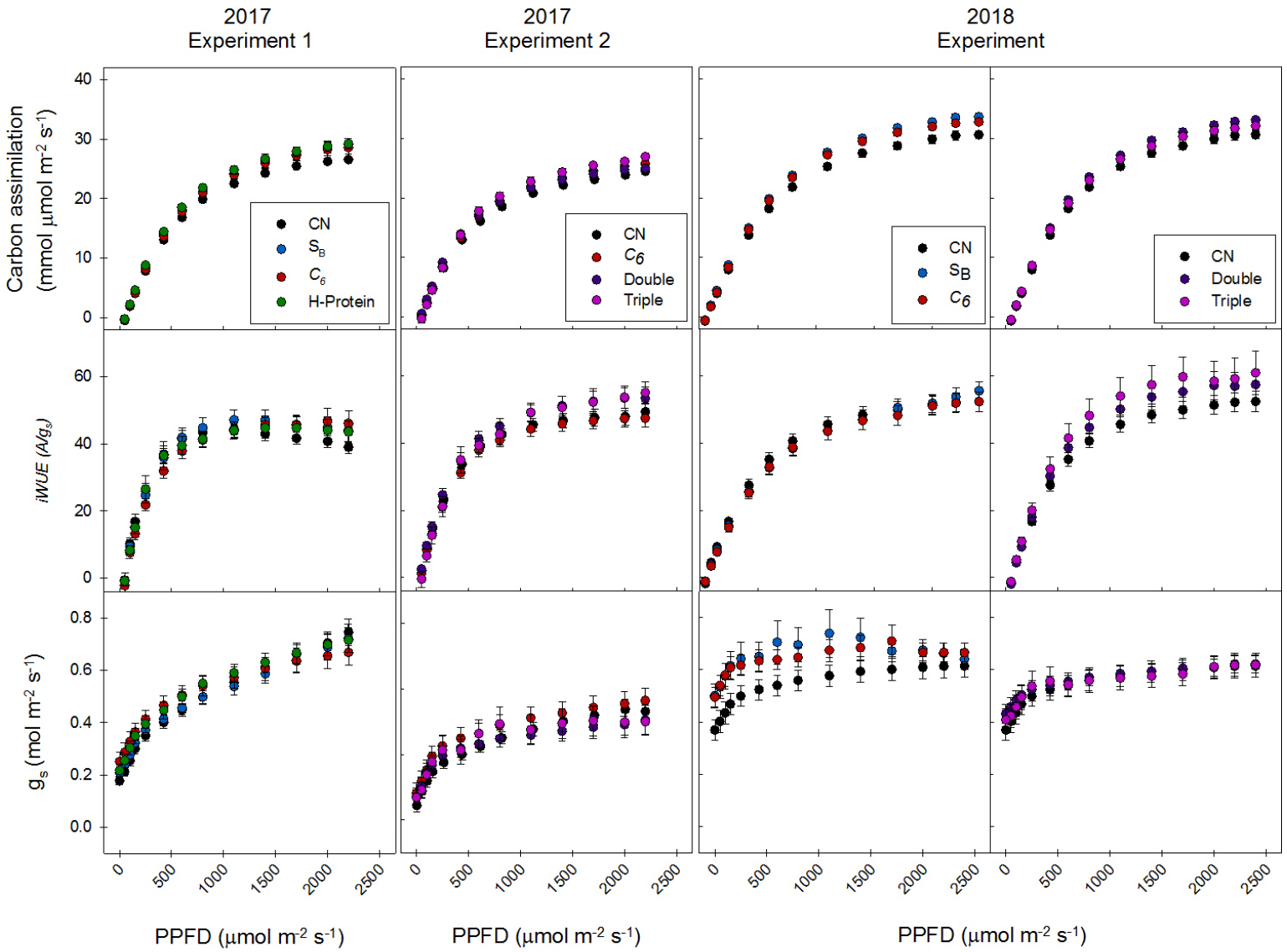

2017
Experiment 1
2017
Experiment 2
2018
Experiment
Carbon assimilation
(mmol µmol m-2 s-1)
iWUE (A/gs)
gs (mol m-2 s-1)
CN
SB
C6
H-Protein
Double
Triple
PPFD (µmol m-2 s-1)
PPFD (µmol m-2 s-1)
PPFD (µmol m-2 s-1)

Figure 5.7. Simultaneous expression of multiple genes can increase water use efficiency under field conditions, over multiple years. (a) Net CO_2 assimilation rate (*A*), (**b**) Stomatal conductance (g_s), (**c**) Intercellular CO_2 concentration (C_i), and (**d**) Intrinsic water-use efficiency (i*WUE*) as a function of light (PPFD) in field-grown plants. Lines expressing cytochrome c_6 (C_6) and FBP/SBPase and cytochrome c_6 (S_BC_6). Control group (CN) represent both WT and azygous plants. Mean ± SE presented. Evaluations are based on 4-5 individual plants from 2-3 independent transgenic lines per manipulation. A linear mixed-effects model and type III ANOVA and contrast analysis to determine significance.

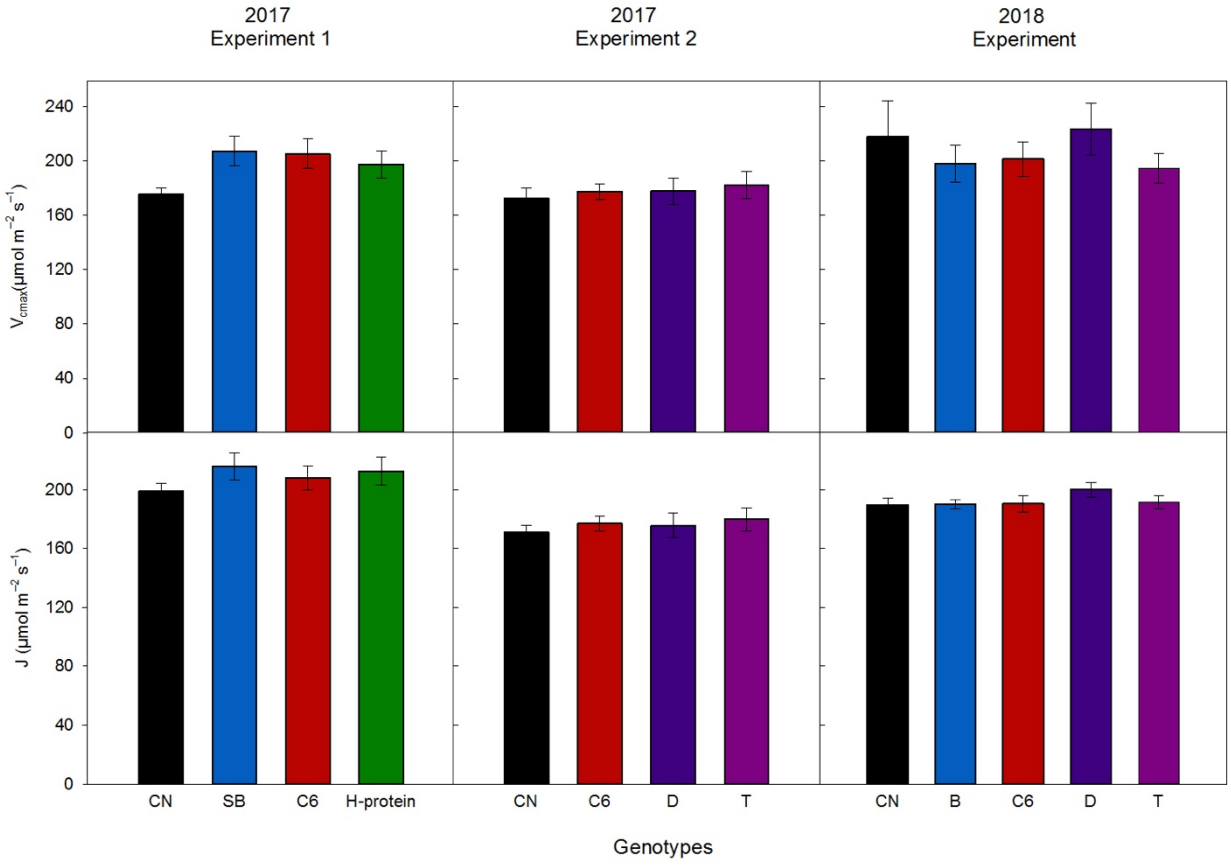


Figure 5.8. No difference is observed in V_{cmax} or J in field grown plants. CO_2 responses curves were modelled (Sharkey, 2016) to obtain the predicts values of V_{cmax} and J at 25 ^{o}C. An ANOVA was used to determine significances. There was no significant difference between the transgenic lines and the control plants for either year. Error bars represent standard error

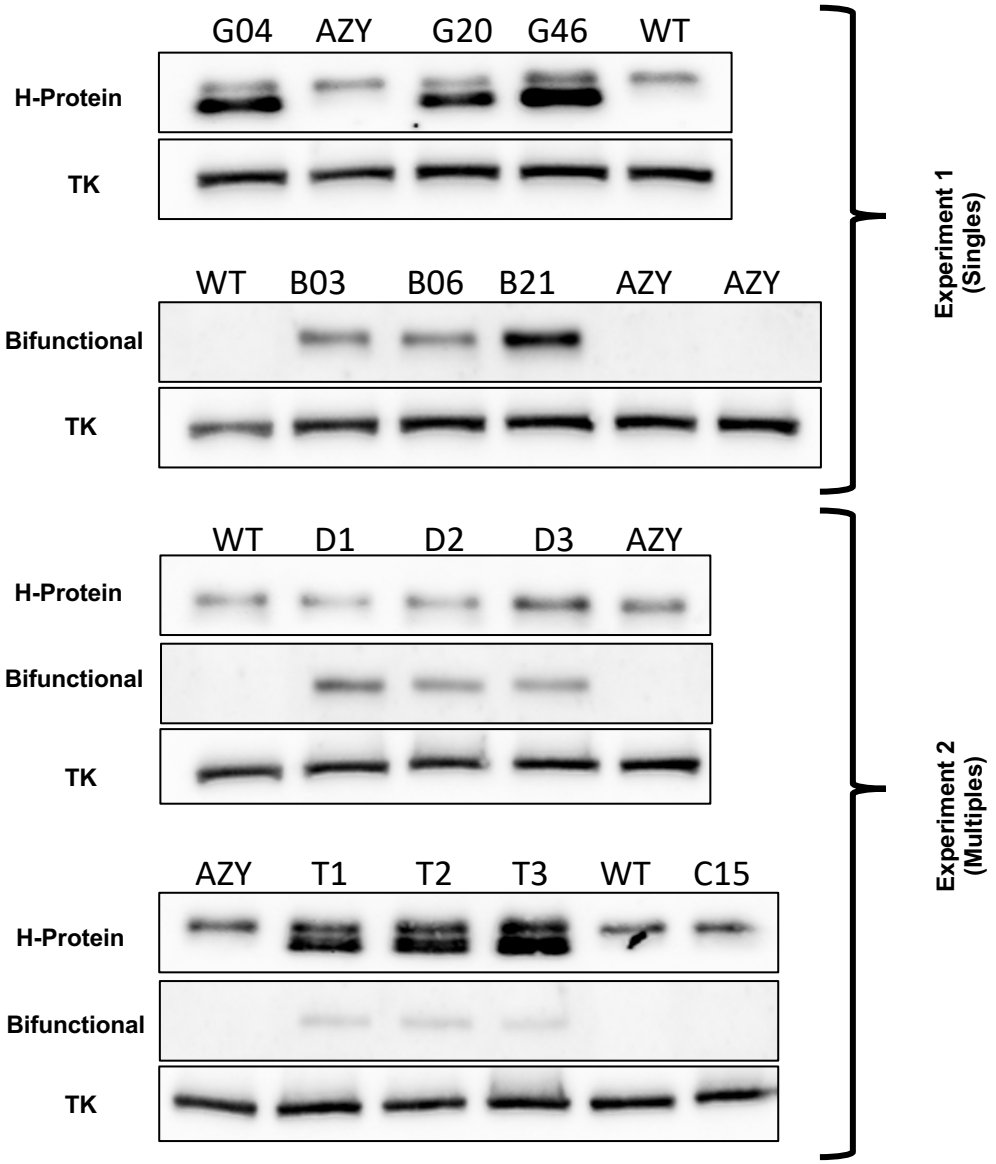


Figure 5.9. Immunoblot analysis from leaf disks using custom raised antibodies. Immunoblot analysis of protein extracts from the newest fully expanded leaf of field grown transgenic and wildtype plants. Approximately 8 µg of protein was separated on a 12 % polyacrylamide gel and blotted onto nylon membrane and probed with polyclonal antibodies against the Bifunctional FBP/SBPase, H-protein (Timm *et al.,* 2012) and TK. A nitrocellulose membrane with a 0.45 µm pore size was used during the transfer of proteins.

Discussion

This chapter looks to expand on the work completed in the 2016 trial, by phenotyping plants with multiple genes targeted in the genotype, not just single manipulations. This was achieved by the expression of S_B and *C_6* in combination or the triple manipulation consisting of S_B, *C_6* and H-protein, which provided the largest increases to biomass, in the 2017 season with increases of ~18 %. The manipulations to these plants were designed to improve productivity, in terms of total biomass, by increasing RuBP regeneration.

5.5 Effects of increasing RuBP regeneration of growth

During the previous trials that were conducted in 2016, the overexpression of the H-protein resulted in an increase in biomass of ~30 %. The H-protein plants were grown again in experiment 1 of the 2017 field season and a significant difference was seen in biomass in two out of three lines when compared to control. This increase was ~25 % for two of the lines, while the third line showed no change over the controls. Although this was less than that observed in 2016, it provides a clear demonstration of the positive effect of overexpression of the H protein over two seasons. In fact, the change in biomass was consistent with glasshouse studies overexpressing this protein where an increase in biomass of between 5 and 40 % was observed, but with only one out of three lines being significantly bigger than the control plants (Lopez-Calcagno et al., 2019). A similar result was observed when overexpressing the H-protein in *Arabidopsis thaliana*, with plants growing to ~120 % of the control group (Timm et al., 2012). As with the biomass data, both this experiment and previous studies (Timm et al., 2012, Lopez-Calcagno et al., 2019) demonstrated how the overexpression of the H-protein leads to higher assimilation rates (Fig. 5.7).

The overexpression of the S_B, D and T manipulations resulted in an increase in biomass each year, but the differences were not significant for the data collected in the 2018 trials. The lines expressing the S_B gene had a varied growth phenotype. Of the four lines used in the experiments, only one of the lines seemed to have a consistent increase in growth, although this was not significant in 2018. The remaining three lines performed as the CN group. The data collected in these experiments corresponds to the observed phenotype of growing SBPase overexpressing lines in unaltered environmental conditions, where a consistent increase in growth has not been seen (Kohler et al., 2017, Rosenthal et al., 2011).

Soybean expressing the S_B gene was grown through three years of field trials in heated FACE (Free Air Concentration Enrichment) experiments. Plants grown under ambient conditions show a significant increase in yield in only one of the three years (Kohler et al., 2017). In results obtained from this study, the trial mirrors the differences in growth observed in the plants expressing the S_B protein. Although, when the CN group and the S_B group of soybean were grown in elevated CO_2 and elevated heat, Kohler et al. (2017) observed a consistent increase in yield over the three years.

For tobacco plants that were overexpressing SBPase, grown in elevated CO_2 field trials, the results match the findings of Kohler et al. (2017). When the plants were grown in ambient conditions, no difference could be observed, but when grown in elevated CO_2, SBPase overexpressing plants had a significant growth phenotype (Rosenthal et al., 2011). All together, these results provide evidence that there is potential for using overexpression of the SBPase protein to improve yield with the most consistent results being observed under elevated CO_2. This is perhaps not surprising as under these conditions RuBisCO limitation will be reduced and therefore control of carbon flux in the

C3 cycle will shift. Looking at this, along with evidence from modelling, there has been suggested that SBPase would be important in these conditions (Zhu et al., 2007, Zhu et al., 2010).

The observed changes in biomass for the D and T genotypes were more pronounced than the single S_B plant, over two years. When all genotypes were grouped for each manipulation, S_B plants had an increase in ~7 % in 2017 and ~4 % in 2018. The D and T manipulations had an increase in biomass over the S_B plants. In the 2017 experiment, the D and T manipulations had an increase in harvest of ~17 and ~18 %, respectively. This increase was seen again in the 2018 trial, albeit a smaller increase of ~8 % for D and ~5 % for T lines.

One notable observation was the range of about 4-fold difference in biomass data obtained from the CN plants in each of the experiments. This implied that there is a substantial relationship between environmental conditions and the growth of *N.tabacum*. Interestingly, there is no discernible difference in the biomass phenotype between the C_6 plant and CN plants in any of the three experiments. The large range in final biomass suggests how the plants are growing in the presence of stress that seriously affect the maximum growth of the control plants. These variation in growing conditions add robustness to the differences that are observed in each year, as these differences are present even when growing under substation stress.

The difference in growth can be seen in total biomass, although not in the plants developmental stage. The difference in leaf number was ~1 between all the experiments, which is very little considering plants will be losing leaves in bad weather. The largest difference observed in our experiments was between 2017 and 2018. It is difficult to be certain of the underlying cause; the weather conditions, water availability or soil quality.

With the uncertainty about what the root cause of the variation in growth, it makes it difficult to mitigate the problem.

5.6 Effects of increasing RuBP photosynthetic capacity

Instantaneous measurements of photosynthesis suggest that an increase in carbon assimilation is not always the driver for the observed differences in biomass. During the 2017 trial, D and T plants had increases in biomass of ~18 %, with only a marginal increase to the assimilation and these increases not being significant (Fig. 5.7). In the 2018 trials however, the double and triple manipulations had an increase in biomass of <10 %. The assimilation rates had a larger increase, with the increase in the D plants being significant. Interestingly, although C_6 did not provide an increase to biomass, it exhibited an increase in Vc_{max}.

Overall, two trends are consistently observed. Firstly, carbon assimilation is increased in each year, yet this increase in not always significant. The reasons for this could be that there is no increase, but as there is a significant increase in carbon assimilation in glasshouse studies (Lopez-Calcagno et al., 2019, Lopez-Calcagno et al., Unpublished), the insignificance may be due to the large variation of daily growing conditions the plants are exposed to. The methodology for obtaining gas exchange data in the glasshouse is to have a standardised leaf temperature (Simkin et al., 2017, Lopez-Calcagno et al., 2019). Working with a consistent leaf temperature will reduce the variation in photosynthetic rate throughout the day. However, due to the conditions in the field where the ambient temperature ranges >10^{o}c in a single day, it is not possible to have a Li-Cor 6400 (LI-COR Biosciences) maintain a standardised leaf temperature. As there is this

range in temperature, and therefore assimilation rates, it is not surprising that the small differences in photosynthesis are not significant.

As well as carbon assimilation, our data suggests that intrinsic water use efficiency (*iWUE)* may be a determinant of total biomass. In both 2017 and 2018, the C_6 had slight increases in carbon assimilation, but the *iWUE* and biomass measurements were no different from the CN group. When the three S_B genotypes are reviewed together, the S_B lines had a marginal increase in *iWUE* and a marginal increase in biomass. The largest differences in biomass were observed the in double and triple manipulation, and correspondingly, the largest differences in *iWUE* were observed by the same lines, in both 2017 and 2018 experiments. Interestingly, this change derived form a different artefact in each year although in both years the D and the T manipulations had an increase in *iWUE*. In 2017 the driver of an increase in *iWUE* was a reduced stomatal conductance (g_s), whilst carbon assimilation was equal to the CN groups. However, in the 2018 trials the increase in *iWUE* was due to a combination of a slight increase in carbon assimilation, paired with a marginal decrease in g_s. Previous field trials, measuring plants that reduce g_s through increasing the Photosystem II subunit S (*PsbS*), provide more evidence that plants can have genetic manipulations to improve water use efficiency, by targeting parts of the photosynthetic pathway. These plants had improved *iWUE* of ~25%, with no detectable difference to yield (Glowacka et al., 2018). The reason for the increase in *iWUE* was due to the manipulation supressing stomatal opening, and as shown in our experiments, this had no impact of carbon assimilation.

The largest difference in biomass was seen in the double and triple manipulations. This is paired with slight increases in both assimilation and *iWUE*. Although the differences in *iWUE* were not significant, the trend seems to be prevalent both years. If there is indeed

an increase in *iWUE*, there would be huge implications. Agriculture is responsible for around 90% of freshwater usage (Scanlon et al., 2012), and a large proportion of this comes from ground water putting pressure on water sources (Dalin et al., 2017). This pressure will only be exacerbated with the impacts of climate change, and so producing plants that consume less water while having higher yield will be most beneficial to the least developed nations; nations that are affected by water shortages.

The hypothesis addressed in this chapter considers how 'multigene manipulations will have led to a cumulative increase in biomass'. The 2018 experiment had the S_B, D and T manipulations growing in the same experiment. A reason for this was to directly compare the manipulations' performance in comparison to each other and not just the CN group. However, this experiment showed no significant difference between the multigene and single manipulations. In contrast, during the 2017 experiments, S_B plants showed an increase in biomass of 7 %, whereas for the D and T manipulations the increase was 17 and 18 % respectively. It is worth noting that there were two separate experiments in 2017 and the S_B manipulation was grown in experiment 1, while the D and T manipulations were grown in experiment 2, and thus cannot be directly compared. Although no definitive conclusion can be made, comparing these results with both the CN and C_6 lines in 2017 suggests that the double and triple manipulations do have a cumulative increase in productivity over the single gene manipulation, albeit with no detectable difference between the double and triple.

Chapter 6 – General discussion

6.1 The overall rational and aims of the project

The aim of this study was to investigate how plants with manipulations to photosynthesis performs under field conditions, and whether the expression of two and three proteins being expressed in conjunction with each other improves the impact on the plant. This work concludes that improving RuBP regeneration can lead to an increase in biomass, when grown in field conditions. Although there is clear variation in the results obtained from one year to the next, this study has demonstrated consistent increases in biomass in plants expressing a combination of genes.

The underpinning research leading to this study can be traced back as far as the late 1980's and 1990's. Antisense experiments designed to downregulate the individual genes within the C3 cycle demonstrated that more than RuBisCO has a control on the flux of carbon through the pathway (Raines, 2003). The advancements in technology since these studies were conducted means there is no longer a sole focus on downregulation experiments to determine the importance of each gene. The first papers upregulating the genes within the C3 cycle confirmed what had been predicted in previous years, that overexpressing genes within the photosynthetic pathways can produce higher yielding plants (Miyagawa et al., 2001, Lefebvre et al., 2005, Feng et al., 2007, Tamoi et al., 2006). Since then, more has been learned about the complexity of the flux of carbon through C3 cycle. This flux is not controlled by a single protein, but by several at any given environmental condition (Zhu et al., 2008). The validity has been demonstrated with experiments that have manipulated multiple enzymes associated with carbon fixation. It has been shown several times that multiple manipulations can have an accumulative effect of the productivity of plants (Simkin et al., 2017, Simkin et al., 2015). However, the work carried out on these plants to date has been conducted in glasshouse conditions. Trials run in controlled environmental conditions have provided invaluable data to

progress this topic. Glasshouse conditions are highly controlled and relatively stable, and thus do not truly reflect how plants grow in a natural environment. To test how plants will perform in a natural environment and get a better representation of how the manipulated genes will function in a natural environment, it is necessary to grow them in the said environment.

As negative results rarely, if ever, get published, it is difficult to gain access to these findings. There is an exception to this with SBPase. Plants overexpressing this protein, or the bifunctional FBPase/SBPase protein, have been trialled in FACE experiments where plants growing under prevailing environmental conditions are used as a control. This has demonstrated how the tobacco plants overexpressing SBPase has no change in biomass relative to CN plants (Rosenthal et al., 2011) when grown in natural environmental conditions. However, in elevated CO_2, a positive effect on both photosynthesis and biomass is observed. Soybean expressing the bifunctional FBP/SBPase gene, growing in natural environmental conditions, demonstrated yield increases (Kohler et al., 2017). Yet, this increase was seen in only one out of three years. This result demonstrates how growth environments can have an impact on how plants with altered carbon metabolism respond. As glasshouse experiments involving plants with several manipulations to carbon metabolism demonstrate an accumulative impact on productivity, would these plants perform more consistently in field conditions?

To investigate the aims of this project a range of transgenic plants, expressing a single gene, were made. Independent lines (50) were made for each construct and several factors were considered to select for suitable plants. These included transgene insertion events, transcript levels and protein expression. In this experiment, plants were selected based on their molecular phenotype, before being tested for a physiological response. An

alternative approach would select plants from tissue culture with the desired phenotype and only discard those where the molecular characterisation does not show the expression of the introduced transgene. This approach was taken by Kromdijk et al. (2016) who used a chlorophyll fluorescence phenotype to select plants that performed as expected based of theory of more rapid NPQ relaxation. Deciding on the best approach to select transgenic plants can be challenging. If producing a plant with the desired genotype does not translate to the predicted phenotype, is that an issue with the plant or the prediction? By selecting plants based on the molecular characteristics you reduce the chance of being biased with the selection of plants, but by selecting plants based on the phenotype, any plant that was adversely affected by the transformation process will be removed. Ultimately, the plants in this experiment were selected based on the genotype to reduce the chance of being biased in the selection process and this method led to some plants not having the desired phenotype, *i.e.* S_B6 (Fig. 6.1). Although, by not back filling a genotype to a preselected phenotype, it is assumed that this to be a more sincere representation of the impact of expressing these genes have.

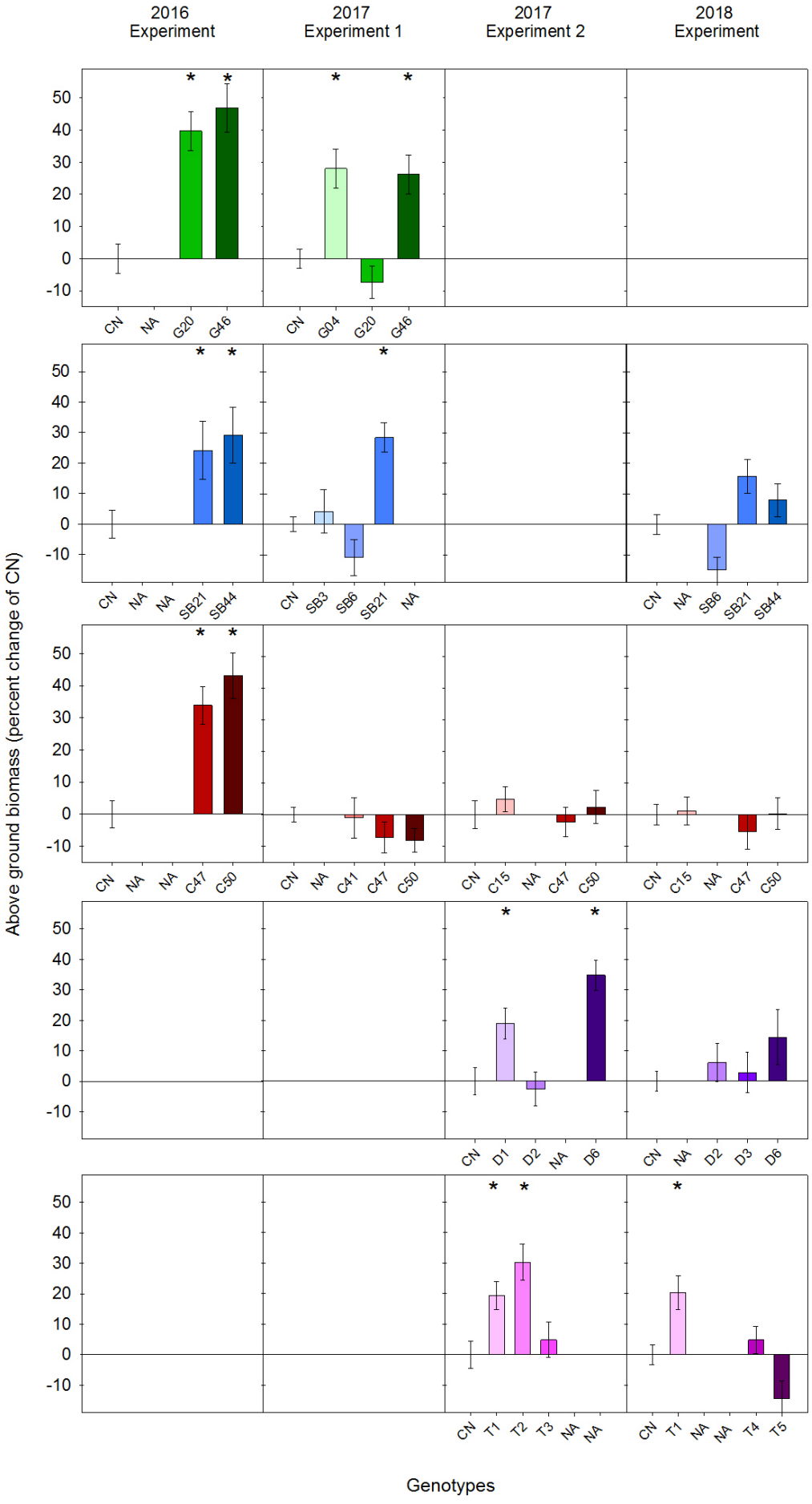

2016 Experiment
2017 Experiment 1
2017 Experiment 2
2018 Experiment
Above ground biomass (percent change of CN)
Genotypes
CN NA G20 G46
CN G04 G20 G46
CN NA NA SB21 SB44
CN SB3 SB6 SB21 NA
CN NA SB6 SB21 SB44
CN NA NA C47 C50
CN NA C41 C47 C50
CN C15 NA C47 C50
CN C15 NA C47 C50
CN D1 D2 NA D6
CN NA D2 D3 D6
CN T1 T2 T3 NA NA
CN T1 NA NA T4 T5

Figure 6.1. Expression of genes led to a varied growth phenotype between the line and through put the four experiments. In the 2016 experiment plants were germinated and seedling grew in the greenhouse for 26 days before being transplanted into the field for further 14 days and then harvested. Control n=44. transgenic bars n=11.

In 2017 and 2018 tobacco plants were germinated in growth cabinets and moved to the glasshouse at 10-14 d post-germination, where seedling were transplanted into hydroponic trays. The plants were transplanted into the field trials ~20 days after germination. The plants were harvested once the canopy was closed and over 50 % of the plants were flowering. Plants were harvested and plant height, leaf area and above-ground dry biomass (TDW) were determined. Control group represent both WT and azygous plants. Mean and SE presented. 2017 control n=144. 2017 transgenic groups n= 72 individual plants from 3 independent transgenic lines. 2018 control n= 64. 2018 transgenic groups n= 96 individual plants from 3 independent transgenic lines. Bars labelled NA are place holders for genotypes not used in the experiment. *indicated a p value of 0.05 or below

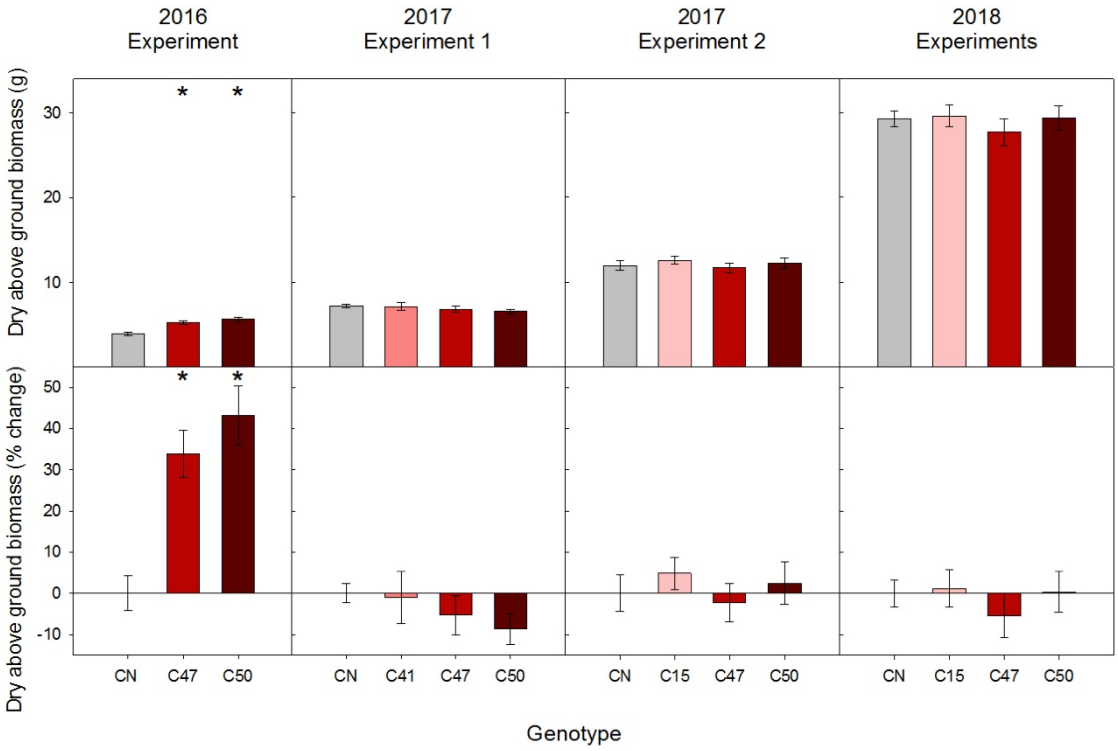


Figure 6.2. Plants expression cytochrome c6 have a varied growth phenotype each year. In the 2016 experiment plants were germinated and seedling grew in the greenhouse for 26 days before being transplanted into the field for further 14 days and then harvested. Control n=44. transgenic bars n=11. 2017 control n=144. 2017 transgenic groups n= 72 individual plants from 3 independent transgenic lines. 2018 control n= 64. 2018 transgenic groups n= 96 individual plants from 3 independent transgenic lines. Bars labelled NA are place holders for genotypes not used in the experiment. *indicated a p value of 0.05 or below

6.2 The impact of genetic manipulations to plant productivity

Altering RuBP regeneration by manipulating enzymes involved in the photosynthetic pathways provides increases to the plants growth (Fig. 6.1). However, deciding what manipulation provides the best improvement to productivity is difficult to determine.

The yearly differences in growth conditions and how the transgenic plants grow in comparison to CN plants indicate there may not be a gene or combination of genes that are ideal for every environmental condition. The work of Kohler et al. (2017) with soybean overexpressing SBPase, highlighted the inconsistent relative growth between transgenic plants and CN plants for each year. Under ambient conditions, overexpressing SBPase does not have a consistent increased yield in soybean (Kohler et al., 2017). This inconsistency was clearly demonstrated with the tobacco plants overexpressing the bifunctional FBP/SBPase in these experiments (Fig. 6.1). As with the S_B plants, *C_6* also had an inconsistent phenotype. In both 2017 experiments and the 2018 season the plants were almost identical to the CN group. However, the *C_6* plants did have an increase in biomass of 35-45 % in the 2016 field season (Fig. 6.2). These inconsistencies could suggest an issue with the CN plants or an unconscious bias favouring the transgenic plants in these experiments. Although, the experiments (Kohler et al., 2017), as well as the experiments in this study, highlight the inconsistency over several seasons. However, during the 2016 field trials the plants were harvested at an earlier developmental stage. This could suggest that the manipulation is having a benefit to growth early on, but this affect is lost later in development.

Glasshouse experiments have shown that transgenic plants with multiple manipulations to the photosynthetic pathways are more productive than plants expressing a single gene

(Simkin et al., 2017, Simkin et al., 2015, Lopez-Calcagno et al., Unpublished). One of the aims of this project was to determine if multigene manipulations are more productive than single gene manipulations in field conditions. Although the results obtained were somewhat inconsistent, evidence was obtained showing that it is possible to confirm this. The uncertainty was caused due to the lack of significant results from the 2018 trial. The 2018 experiment was designed to have the single S_B and *C6*, the double S_B+*C6* and the Triple S_B+*C6*+H-protein manipulations grown together, and thereby it would be possible to directly compare the manipulations. However, the results from 2018 did not provide a definite answer with the data obtained. In fact, only one line (T1) (Fig. 6.1) was significantly changed from the CN lines. While the 2018 experiment did not show a clear result, the 2017 experiments seemed more conclusive. The biomass of both the *C6* and H-protein manipulations were not significantly bigger than the CN, S_B had increases of ~8 % and the double and triple manipulations had increases of ~18 %. The difference in growth between the single and multigene manipulations seems to give a virtuous answer to the question, as 10 % is an extremely large increase. Nevertheless, it is worth noting that these results were obtained from two different experiments in 2017, and so cannot be directly compared. In the 2017 experiments only *C6* plants were grown in both field trials. The *C6* plants performed equally in both these experiments, thus the 2017 the multigene manipulations may be concluded as more productive than single gene manipulation and CN plants.

Looking at the entire growth data, across the four experiments, it is clear how increasing RuBP regeneration via the manipulation of proteins involved with in photosynthesis leads to a more productive plant in the field. There is also strong evidence suggesting that multiple manipulations have a larger impact than single manipulations.

Although higher yielding plants were produced, there was a large range in biomass between lines in each year (Fig 6.1). Trying to determine the cause of this variation may prove useful for furthering the scientific communities understanding on what leads to higher yielding plants. There could be several causes of the variation, including damage to individuals from pest of adverse weather. However, the cause of inconsistent growth may also be linked to the amount of the protein that was introduced. A range of protein amounts in different lines is demonstrated in Fig 4.8 and Fig 5.9, however, due to the methodology used to present the immunoblots, it is not possible to accurately quantify these figures. Taylor and Posch (2014) provides a guide on how to quantify immunoblots and moving forward, implementing this methodology would provide additional information on the molecular characteristics of the transgenic plants that could yield interesting data on the topic.

6.3 The larger implications of this work and how to progress the findings

This project was funded to help secure a future free from malnutrition. With the expected need for increased calorie demands (Tilman et al., 2011) it is important to tackle the issue of food shortages on many fronts. Working to improve crop productivity is one of the ways that could help future generations avoid a lack of food. In recent years there has been breakthroughs in photosynthetic research that could provide more productive plants. Some of the most notable experiments have come with manipulating photosynthesis but taking a different approach to achieve this. Kromdijk et al. (2016) produced plants that increase the rate of which plants recover from photoprotection. By producing plants that can more efficiently deal with fluctuating light environments, the dry biomass of tobacco increased by 15 %. Another approach (South et al. 2019) looked at producing a synthetic pathway that bypass photorespiration. These introduced pathway produced tobacco plants that were up to 40 % more productive (South et al., 2019). The work of this study

across the three years of field trials has also demonstrated increases of up to 40% in dry biomass. Manipulating RuBP regeneration can be moved forward in several ways, with meticulous trials to determine the best combination of genes. The challenge of this approach is the amount of resources which is required. The alternative approach would be to exploit the work that was already completed in NPQ (Kromdijk et al., 2016) and with that introduce novel bypasses to photorespiration (South et al., 2019). This would be a particularly interesting approach considering each of these projects focuses on a separate part of photosynthesis. With several, plants could be produced that have an increased RuBP regeneration rate, faster relaxing of NPQ and a synthetic pathway that avoids photorespiration.

Increasing the productivity of model species is a good start for realisation of crop improvements, but how will these discoveries translate into crops when we are looking at grain yield and not just trying to grow bigger plants? There has already been some research in single gene manipulations of wheat in glasshouse conditions (Driever et al., 2017), which were positive. However, the real test would be transforming crop species with the multigene manipulations and observing the yield difference in the natural field environment.

6.4 Summary of outcome and their wider relevance

- It was possible to produce large number of independent transgenic plants, with a stable genotype, that were phenotyped in field trials and available for future research.

The ability to streamline the transformation process reduced the time and effort involved in producing transgenic plants, making it easier to test hypotheses.

- Improving RuBP regeneration leads to an increase in photosynthetic efficiencies biomass of field grown plants.

By improving the productivity of plants in helps with the problem of food insecurity. By 2050 it is estimated that a 50 % increase more food, will be needed to feed the world's population (Tilman et al., 2011).

- Manipulating multiple genes involved in carbon fixation had additive effect on growth rates and photosynthetic capacity.

This work demonstrates that manipulating multiple genes involved in carbon fixation can lead to improved photosynthesis and growth. It is now important to understand what combination of genes would have the greatest impact, and in what environments each combination would be most productive.

- The same plants may have a different phenotype in different field seasons

The genotype x experimental interactions appear to change the growth phenotype of individual lines year on year. Determining what levels of gene expression, or what combination of genes expressed, produce the most suited plants for a predicted climate may produce higher yielding plants.

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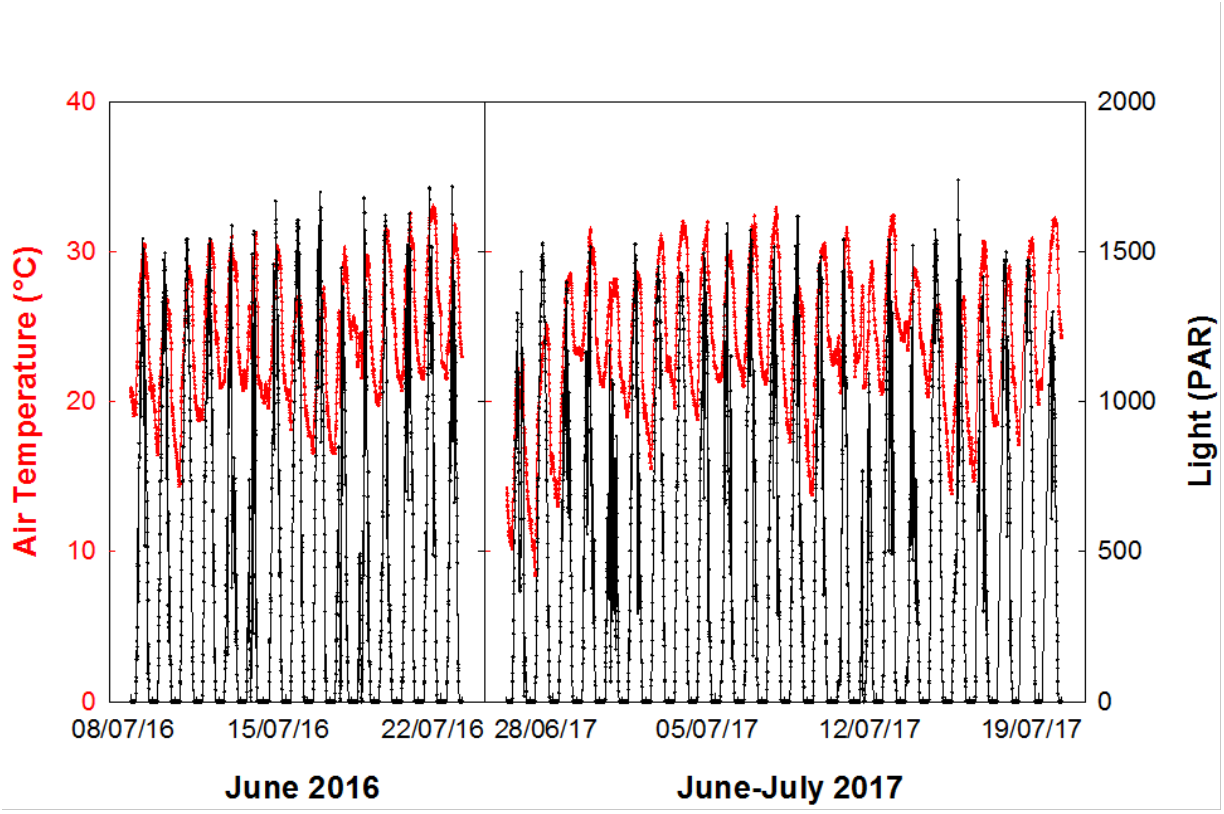
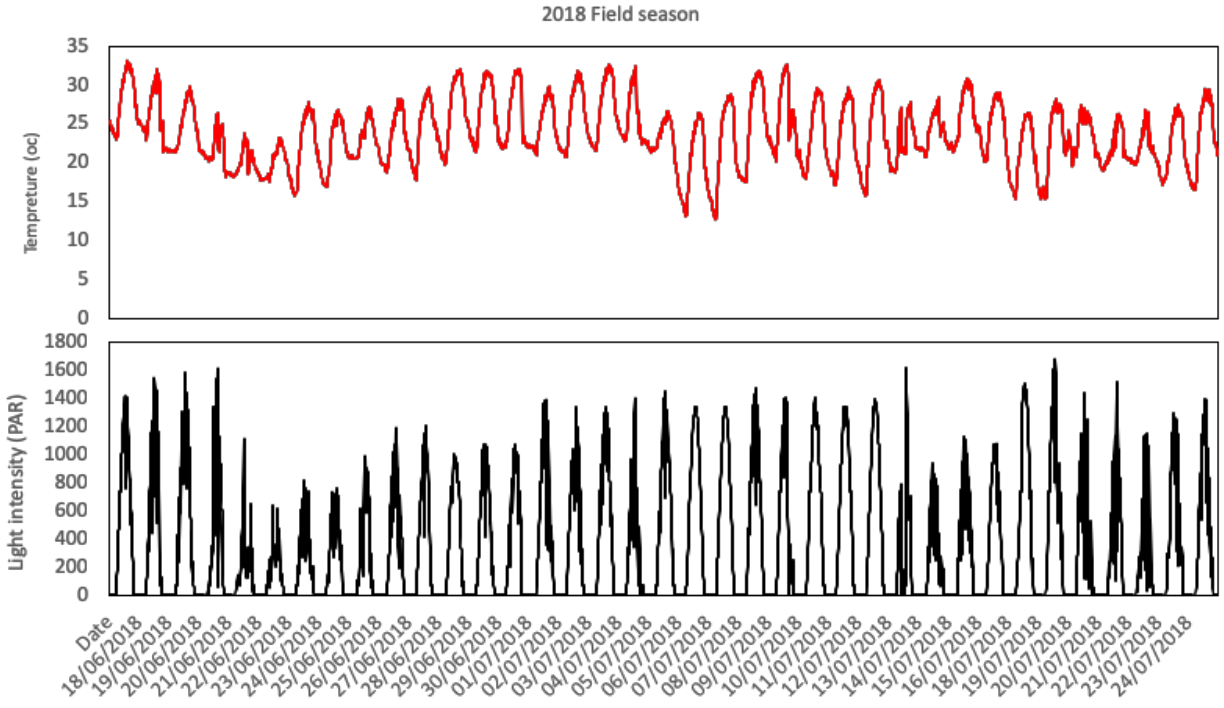
Appendix

Appendix A-Weather station environmental data from 2016, 2017 and 2018





Appendix Figure 1.1. Environmental conditions during field trials. Air temperature and light intensity from 2016, 2017 and 2018 growth season. In 2016 and 2017 data was collected in 30-minute intervals. 2018 data was collected in 10-minute intervals.

Appendix B-Construct I.D. information

Construct I.D.	Entery vector	Selection marker	Promoter	Gene of intrest (position 1)	Promoter	Gene of intrest (position 2)	Promoter	Gene of intrest (position 3)	Promoter	Gene of intrest (position 4)
EC23025	pL2B	Hygromycine B	p35S	AGPase						
EC23028	pL2B	Hygromycine B	p35S	Cytochrome c6						
EC23030	pL2B	Hygromycine B	pFMV	FBP Aldolase						
EC23083	pL2B	Hygromycine B	pFMV	Bifunctional FBPase/SBPase						
EC23084	pL2B	Hygromycine B	pFMV	Bifunctional FBPase/SBPase	pFMV	FBP Aldolase				
EC23085	pL2B	Hygromycine B	pFMV	Bifunctional FBPase/SBPase	pFMV	FBP Aldolase	p35S	Cytochrome c6		
EC23086	pL2B	Hygromycine B	pFMV	Bifunctional FBPase/SBPase	pFMV	FBP Aldolase	p35S	Cytochrome c6	p35S	AGPase
EC23088	pL2B	Hygromycine B	pFMV	H-Protein						
EC23089	pL2B	Hygromycine B	pFMV	H-Protein	pFMV	Bifunctional FBPase/SBPase				

Appendix Figure 1.2. Construct I.D. information. A breakdown of each construct, including the selection marker, gene of interest and the promoter that dives the corresponding gene

Appendix C - Lopez-Calcagno *et al*., Unpublished

Simultaneous stimulation of RuBP regeneration and electron transport increases productivity and water use efficiency under field conditions

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Short title: Improving photosynthesis and yield

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One sentence summary: Simultaneous stimulation of RuBP regeneration and electron transport results in improvements in biomass yield in glasshouse and field grown tobacco.

Abstract

Previous studies have demonstrated that independent stimulation of either electron transport or RuBP regeneration can increase the rate of photosynthetic carbon assimilation and plant biomass. In this paper, we present evidence that a multi-gene approach to simultaneously manipulate these two processes provides a further stimulation of photosynthesis. We report on the introduction of the cyanobacterial bifunctional enzyme fructose-1,6-bisphosphatase/sedoheptulose-1,7-bisphosphatase or overexpression of the plant enzyme sedoheptulose-1,7-bisphosphatase, together with expression of the red algal protein cytochrome c_6, and show that a further increase in biomass accumulation under both glasshouse and field conditions can be achieved. Furthermore, we provide evidence that the simultaneous stimulation of electron transport and RuBP regeneration can lead to enhanced intrinsic water use efficiency under field conditions.

Keywords: SBPase; FBP/SBPase; Calvin-Benson cycle; cytochrome c_6; chlorophyll fluorescence imaging; transgenic; electron transport; biomass; water use efficiency.

Yield potential of seed crops grown under optimal management practices, and in the absence of biotic and abiotic stress, is determined by incident solar radiation over the growing season, the efficiency of light interception, energy conversion efficiency and partitioning or harvest index. The “harvest index” is the amount of total energy partitioned into the harvestable portion of the crop, whilst the “conversion efficiency” is the ratio of biomass energy produced over a given period divided by light energy intercepted by the canopy over the same period[1]. For the major crops, the only component not close to the theoretical maximum is energy conversion efficiency, which is determined by gross canopy photosynthesis minus respiration. This highlights photosynthesis as a target for improvement to raise yield potential in major seed crops[1].

Transgenic experiments and modelling studies have provided compelling evidence that increasing the levels of photosynthetic enzymes in the Calvin Benson (CB) cycle has the potential to impact photosynthetic rate and yield[1-13]. Early experimental evidence illustrated that even small reductions in the CB enzymes sedoheptulose-1,7-bisphosphatase (SBPase[14-16]), fructose-1,6-bisphophate aldolase (FBPA[17,18]) and the chloroplastic fructose-1,6-bisphosphatase (FBPase[19-21]) negatively impact on carbon assimilation and plant growth. These studies indicated that these enzymes exercise significant control over photosynthetic efficiency, suggesting that improvements in photosynthetic carbon fixation may be achieved and maintained through manipulation of CB cycle enzymes[11,22]. These results also demonstrated that, although some enzymes exert more control over the CB cycle than others, there is no single limiting step in photosynthetic carbon assimilation. Over the last two decades a number of transgenic studies have supported this. Over-expression of SBPase in tobacco[3,5,6], Arabidopsis[7], tomato[13] and wheat[23] has demonstrated the potential of manipulating the expression of CB cycle enzymes and specifically the regeneration of RuBP to increase growth, biomass (30-42%) and even seed yield (10-53%). Similarly, overexpression of other enzymes including FBPA[12], cyanobacterial SBPase, FBPase[24] and the bifunctional fructose-1,6-bisphosphatases/sedoheptulose-1,7-bisphosphatase (FBP/SBPase[2,25,26]) in a range of species including tobacco, lettuce and soybean has shown that increasing photosynthesis increases yield, reinforcing the original hypothesis that manipulating the activity of the CB cycle enzymes can be used to increase productivity.

In addition to manipulation of CB cycle genes, increasing photosynthetic electron transport has also been shown to have a beneficial effect on plant growth. Overexpression of the Rieske FeS protein -a key component of the cytochrome *b*$_6$*f* complex- in Arabidopsis, has previously been shown to lead to increases in electron transport rates, CO_2 assimilation, biomass and seed yield[27]. Similarly, the introduction of the algal cytochrome *c*$_6$ protein into Arabidopsis and tobacco[28,29] resulted in increased growth. In these transgenic plants, electron transport rate was increased along with ATP, NADPH, chlorophyll, starch content, and capacity for CO_2 assimilation. Higher plants have been proposed to have lost the cytochrome *c*$_6$ protein through evolution, but in green algae and cyanobacteria, which have genes for both cytochrome *c*$_6$ and plastocyanin (PC), cytochrome *c*$_6$ has been shown to replace PC as the electron transporter connecting the cytochrome *b*$_6$*f* complex with PSI under Cu deficiency conditions[30,31]. There is evidence showing that PC can limit electron transfer between cytochrome *b*$_6$*f* complex and PSI[32], and in Arabidopsis, it has been shown that introduced algal cytochrome *c*$_6$ is a more efficient electron donor to P700 than PC[28]. This evidence suggests the introduction of the cytochrome *c*$_6$ protein in higher plants as a viable strategy for improving photosynthesis.

Previous research has shown that taking a multi-gene approach to increase the levels of more than one enzyme or protein simultaneously can result in a cumulative increase in photosynthesis and biomass yield[6,7,33]. Building on this approach, the work in this paper aims to test the hypothesis that combining an increase in the activity of a CB cycle enzyme, specifically enhancing RuBP regeneration, together with stimulation of the electron transport chain can boost photosynthesis and yield above that observed when these processes are targeted individually. To test this hypothesis *Nicotiana tabacum* plants expressing the cyanobacterial FBP/SBPase or the higher plant SBPase, and the algal cytochrome *c*$_6$ were generated in two different tobacco cultivars. The analysis presented here demonstrates that the simultaneous stimulation of electron transport and RuBP regeneration leads to a significant increase in photosynthetic carbon assimilation, and results in increased biomass and yield under both glasshouse and field conditions.

RESULTS

Production and Selection of Tobacco Transformants

Previous differences observed in the biomass accumulation between Arabidopsis and tobacco overexpressing SBPase and SBPase plus FBPA[6,7] led us to explore the effect of similar manipulations (RuBP regeneration by overexpression of SBPase or introduction of the cyanobacterial FBP/SBPase, together with enhanced electron transport) in on two different tobacco cultivars with very different growth habits: *N. tabacum* cv. Petite Havana and *N. tabacum* cv. Samsun. Sixty lines of cv. Petit Havana, and up to fourteen lines of cv. Samsun were generated per construct and T0 and T1 transgenic tobacco were screened by qPCR and immuno-blot analysis to select independent lines with expression of the transgenes (data not shown).

N. tabacum cv. Petit Havana T2/T3 progeny expressing FBP/SBPase (S_B; lines S_B03, S_B06, S_B21, S_B44) or cytochrome c_6 (C_6; lines C15, C41, C47, C50) and cv. Samsun lines expressing SBPase + cytochrome c_6 (SC_6, lines 1, 2 and 3) were produced by agrobacterium transformation. *N. tabacum* cv. Petit Havana plants expressing both S_B and C_6 were generated by crossing S_B lines (06, 44, 21) with C_6 lines (15, 47, 50) to generate four independent S_BC_6 lines: S_BC1 (S_B06xC47), S_BC2 (S_B06xC50), S_BC3 (S_B44xC47) and S_BC6 (S_B21xC15). Semi-quantitative RT-PCR was used to detect the presence of the FBP/SBPase transcript in lines S_B and S_BC_6, cytochrome c_6 in lines C_6, S_BC_6 and SC_6, and SBPase in lines S and SC_6 (**Fig. 1a**). The selected S_B and S_BC_6 lines were shown to accumulate FBP/SBPase protein and S and SC_6 to overexpress the SBPase protein by immunoblot analysis (**Supplementary Fig. 1**). In addition to immunoblot analysis, we analysed total extractable FBPase activity in the leaves of the cv. Petite Havana T2/T3 & F3 homozygous progeny lines used to determine chlorophyll fluorescence and photosynthetic parameters. This analysis showed that these plants (S_B and S_BC_6) had increased levels of FBPase activity ranging from 34 to 47% more than the control plants (**Fig. 1b**). The full set of assays showing the variation in FBPase activities between plants can be seen in supplemental data (**Supplementary Fig. 2**). The S and SC_6 lines were from the same generation of transgenic plants used in a previous study and shown to have increased SBPase activity[6]. Antibodies raised against the *Porphyra umbilicalis* cytochrome c_6 protein identified a unique band in the *P. umbilicalis* protein extract, in *E.coli* expressing the *P. umbilicalis* protein and in the combined protein mix of

lines C15, C41 and C47. No bands were observed in wild type or the azygous control (**Supplementary Fig. 3**).

Chlorophyll fluorescence analysis confirmed that in young plants, the operating efficiency of photosystem two (PSII) photochemistry F_q'/F_m' at an irradiance of 600-650 µmol m^{-2} s^{-1} was significantly higher in all selected lines compared to either WT or null segregant controls (**Fig. 1c, d**). However, the F_q'/F_m' values of the S_BC_6 and SC_6 lines, were not significantly different from the F_q'/F_m' values obtained from the plants expressing individually FBP/SBPase (S_B), cytochrome c_6 (C_6) or SBPase (S).

Stimulation of electron transport and RuBP regeneration increases photosynthetic performance in two distinct tobacco varieties under glasshouse conditions

Transgenic lines selected based on the initial screens described above were grown in the glasshouse, in natural light supplemented to provide illumination between 400-1000 µmol m^{-2} s^{-1}. The rate of net CO_2 assimilation (A) and F_q'/F_m' was determined as a function of internal CO_2 concentration (C_i), in mature and developing leaves of *N. tabacum* cv. Samsun (S and SC_6) and in mature leaves of *N. tabacum* cv. Petit Havana (S_B, C_6 and S_BC_6) (**Fig. 2a**). The transgenic lines displayed greater CO_2 assimilation rates than that of the control (CN) plants. A was 15% higher than the controls in the mature leaves of the SC_6, at a C_i of approximately 300 µmol mol^{-1} (equivalent to current atmospheric [CO_2]) (**Fig. 2b**). The developing leaves of the SC_6 plants also showed significant increases in PSII operating efficiency (F_q'/F_m') and in the PSII efficiency factor (F_q'/F_v'; which is determined by the ability of the photosynthetic apparatus to maintain Q_A in the oxidized state and therefore a measure of photochemical quenching) when compared to control plants (**Fig. 2c**). Interestingly, in mature leaves of the cv. Samsun transgenic plants, the differences in assimilation rates and in the operating efficiency of PSII photochemistry between the transgenic and the CN plants were smaller than in the developing leaves. Only the S transgenic plants displayed a higher average value for F_q'/F_m' and F_q'/F_v' than the CN plants at all CO_2 concentrations measured. In contrast, the mature leaves of SC_6 plants displayed F_q'/F_v' values higher than the control only at C_i levels between 300 and 900 µmol mol^{-1} (**Fig. 2b**).

Similar trends were shown for the *N. tabacum* cv. Petit Havana transgenic plants, which displayed higher average values of A, F_q'/F_m' and F_q'/F_v' than the CN (**Fig. 2a**). In the leaves of the S_BC_6 plants (cv. Petit Havana) these significant increases were similar to the developing leaves of the SC_6 lines (cv. Samsun).

The developing leaves of both the S and SC_6 plants (cv. Samsun) showed a significant increase in J_{max} and A_{max} when compared the control plants (**Table 1**). The mature leaves of the SC_6 transgenics also displayed a significantly higher $Vc_{max,}$ J_{max} and A_{max} than the CN. In contrast, the leaves of the S_BC_6 plants (cv. Petite Havana) only had significant increases in A_{max}, although higher average values for $Vc_{max,}$ and J_{max} were evident. These results showed that simultaneous stimulation of electron transport and RuBP regeneration by expression of cytochrome c_6 in combination with FBP/SBPase or SBPase has a greater impact on photosynthesis than the single manipulations in all plants analysed.

Stimulation of electron transport and RuBP regeneration stimulates growth in two distinct tobacco varieties under glasshouse conditions

In parallel experiments, plants expressing FBP/SBPase (S_B), cytochrome c_6 (C_6) and both (S_BC_6) (*N. tabacum* cv. Petite Havana) and plants expressing SBPase (S) and SBPase + cytochrome c_6 (SC_6) (*N. tabacum* cv. Samsun) were grown in the glasshouse for four and six weeks respectively before harvesting. Height, leaf number, total leaf area and above ground biomass were determined (**Fig 3** and **Supplementary Fig 4**). All of the transgenic plants analysed here displayed increased height when compared to CN plants. Plants expressing cytochrome c_6 (C_6, S_BC_6, (cv. Petite Havana) and SC_6 (cv. Samsun)) had a significant increase in leaf area and in stem and leaf biomass compared to their respective controls. In the S_B transgenic plants (cv. Petite Havana) only the biomass of the stem was greater than the CN plants. Notably the S_BC_6 and SC_6 transgenics displayed significantly greater leaf area than the single S_B and S transgenic plants respectively. The total increase in above ground biomass when compared to CN group was 35% in S_B, 44% in C_6 and 9% in S, with consistently higher means in the double manipulations S_BC_6 (52%) and SC_6 (32%).

Simultaneous expression of FBP/SBPase and cytochrome c_6 increases growth and water use efficiency under field conditions.

To test whether the increases in biomass observed in these transgenic plants under glasshouse conditions could be reproduced in a field environment, a subset of lines was selected for testing in the field. Since the larger percentage increases in biomass were displayed by the manipulations in *N. tabacum* cv. Petit Havana, these plants were selected and tested in three field experiments in two different years (2016 and 2017).

In 2016, a small-scale replicated control experiment was carried out to evaluate vegetative growth in the field, in the lines expressing single gene constructs for FBP/SBPase (S_B) and cytochrome c_6 (C_6). Plants were germinated and grown under controlled environment conditions for 25 d before being moved to the field. After 14 d in the field, plants were harvested at an early vegetative stage and plant height, total leaf area and above ground biomass were measured (**Fig 4a**). These data revealed that the S_B and C_6 plants showed an increase in height, leaf area and above ground biomass of 27%, 35% and 25% respectively for S_B and 50%, 41% and 36% respectively for C_6 when compared to CN plants.

In 2017, two larger scale, randomized block design field experiments were carried out to evaluate performance in the S_B, C_6 and S_BC_6 plants compared to CN plants. Plants were grown from seed in the glasshouse for 33 d, and then moved to the field and allowed to grow until the onset of flowering (further 24-33 d), before harvesting. In **Fig 4b, c** it can be seen that the S_B and C_6 plants harvested after the onset of flowering did not display any significant increases in height, leaf area or biomass. Interestingly, plants expressing both FBP/SBPase and cytochrome c_6 (S_BC_6), displayed a significant increase in a number of growth parameters; with 13%, 17% and 27% increases in height, leaf area and above ground biomass respectively when compared to controls.

Additionally, in the 2017 field experiments A as a function of C_i at saturating light (A/C_i) was determined. In the 2017 experiment 1 (Exp.1) a significant increase in A was observed in S_B and C_6 plants without differences in PSII operating efficiency (F_q'/F_m') (**Fig. 5a**). However, in the 2017 experiment 2 (Exp.2), no differences in A or in F_q'/F_m' values were evident in the C_6 and S_BC_6 plants when compared to the CN plants (**Fig. 5b**). Analysis of A as a function of light (A/Q) showed either small or no significant differences

in *A* between genotypes (**Fig. 6a** and **Supplementary Fig 5a**). Interestingly, g_s in the S_BC_6 plants were significantly lower than C_6 and CN plants at light intensities above 1000 μmol m^{-2} s^{-1} (**Fig 6b**), which resulted in a significant increase in intrinsic water use efficiency (*iWUE)* for S_BC_6 plants (**Fig 6d**). No significant differences in *iWUE* were observed for S_B or C_6 transgenic plants (**Fig 6d** and **Supplementary Fig 5d**).

DISCUSSION

In this study, we describe the generation and analysis of transgenic plants with simultaneous increases in electron transport and improved capacity for RuBP regeneration, in two different tobacco cultivars. Here we have shown how independent stimulation of electron transport (by expression of cytochrome c_6) and stimulation of RuBP regeneration (by expression of FBP/SBPase or overexpression of SBPase) increased photosynthesis and biomass in glasshouse studies. Furthermore, we demonstrated how the targeting of these two processes simultaneously (in the S_BC_6 and SC_6 plants) had an even greater effect in stimulating photosynthesis and growth. Additionally, in field studies we demonstrate that plants with simultaneous stimulation of electron transport and of RuBP regeneration had increased *iWUE* with an increase in biomass.

Under glasshouse conditions, increases in photosynthesis were observed in all of the transgenic plants analysed here and these were found to be consistently correlated with increases in biomass. Although increases in photosynthesis and biomass have been reported for plants with stimulation of RuBP regeneration in both model[2,3,6,7,33] and crop[25,23] species; and electron transport in Arabidopsis and tobacco[27-29], the data presented here provides the first report of increased photosynthesis and biomass by the simultaneous stimulation of electron transport and RuBP regeneration. Increases in A were observed under glasshouse conditions in the leaves of all of the different transgenic tobacco plants and in both of the tobacco cultivars (cv. Petit Havana and cv. Samsun). Analysis of the A/C_i response curves showed that the average values for the photosynthetic parameters Vc_{max}, J_{max} and A_{max} increased by up to 17, 14 and 12% respectively. These results indicated that not only was the maximal rate of electron transport and RuBP regeneration increased, but the rate of carboxylation by Rubisco was also increased. Although this may seem counterintuitive in that we have not targeted directly Rubisco activity, it is in keeping with a study by Wullschleger[34] of over 100 plant species that showed a linear correlation between J_{max} and Vc_{max}. Furthermore, it has also been shown previously that overexpression of SBPase leads not only to a significant increase in J_{max} but that an increase in Vc_{max} and Rubisco activation state[3,6].

Notably, in the greenhouse study, the highest photosynthetic rates were obtained from the leaves of plants in which both electron transport and RuBP regeneration (S_BC_6 and SC_6) were boosted, suggesting that the co-expression of these genes results in an additive effect on improving photosynthesis. In addition to the increases in *A*, the plants with simultaneous stimulation of electron transport and RuBP regeneration displayed a significant increase in F_q'/F_m', indicating a higher quantum yield of linear electron flux through PSII compared to the control plants. These results are in keeping with the published data for the introduction of cytochrome c_6 and the overexpression of the Rieske FeS protein in Arabidopsis[27,28]. In both of these studies the plants had a higher quantum yield of PSII and a more oxidised plastoquinone pool[28], suggesting that, although PC is not always limiting under all growth conditions[35], there is scope to stimulate reduction of PSI by using alternative, more efficient electron donors to PSI[28,32]. Furthermore, in the S_BC_6 and SC_6 plants the increase in F_q'/F_m' was found to be largely driven by the increase in the PSII efficiency factor (F_q'/F_v'). This suggests that the increase in efficiency in these plants is likely due to stimulation of processes down stream of PSII such as CO_2 assimilation.

To provide further evidence of the applicability of targeting both electron transport and RuBP regeneration to improve crop yields, we tested these plants in the field. Here we showed that the expression FBP/SBPase alone led to an increase in growth and biomass in the 2016 field-grown plants of between 22-40%, when harvested during early vegetative growth, prior to the onset of flowing. Interestingly, when plants with the same transgenic manipulations were harvested later in development, after the onset of flowering, in the 2017 field trials, this advantage was no longer evident and the single FBP/SBPase expressors were indistinguishable from the control plants. These results are in contrast to the 2016 field data and may be due to the later timing in development of the harvest in the 2017 experiment.

The transgenic plants expressing cytochrome c_6 alone also showed enhanced growth and biomass early development, but as with the FBPase/SBPase plants, this improvement was no longer evident when plants were harvested after flowering. This difference in biomass gain between the early and late harvest was not observed in a parallel experiment, where the overexpression of H-protein was shown to increase

biomass under field conditions in plants harvested in early development and after the onset of flowering[36]. These results suggest that the expression of FBP/SBPase or cytochrome c_6 alone, may provide an advantage under particular sets of conditions or at specific stages of plant development. This might be exploitable for some crops where an early harvest is desirable (eg. some types of lettuce, spinach and tender greens)[25]. In contrast with the results with the single manipulations described above, plants simultaneously expressing both cytochrome c_6 and FBP/SBPase displayed a consistent increase in biomass after flowering under field conditions.

In the transgenic lines grown in the field, the correlation between increases in photosynthesis and increased biomass were less consistent than that observed under glasshouse conditions. The significant increases in photosynthetic capacity displayed by the FBP/SBPase and cytochrome c_6 expressors in 2017 Exp. 1, provided clear evidence that these individual manipulations are able to significantly stimulate photosynthetic performance under field conditions. However, no increase in biomass was evident in these plants. In contrast in the 2017 Exp. 2 we did not detect any significant differences in photosynthetic capacity in either the cytochrome c_6 expressors or the plants with simultaneous expression of FBP/SBPase + cytochrome c_6 expressors, but increased biomass was evident. At this point we have no explanation for this disparity. However, although not significantly different, in all experiments, the mean A values of the transgenic plants were consistently higher than those of the controls. It is known that even small increases in assimilation throughout the lifetime of a plant will have a cumulative effect, which could translate into a significant biomass accumulation[6], this may in part explain the disparity with the biomass results presented.

An unexpected result that was found only in the plants with simultaneous expression of FBP/SBPase + cytochrome c_6 (S_BC_6), was that they had a lower g_s and lower C_i concentration at light intensities above 1000 μmol m^{-2} s^{-1}, when compared to control plants. Normally lower C_i would be expected to lead to a reduction in photosynthesis but, interestingly, these plants were able to maintain CO_2 assimilation rates equal to or higher

than control plants resulting in an improvement in *iWUE*. A similar improvement in *iWUE* was seen in plants overexpressing the NPQ related protein, PsbS[37]. It was shown that light-induced stomatal opening was reduced in these plants which had a more oxidized Q_A pool, which has been proposed to act as a signal in stomatal movement[38]. Our results provide further support for the proposal that the increased capacity for photosynthesis in the S_BC_6 plants is compensating for the reduction in C_i. This higher *iWUE* and the fact that a higher productivity than controls has been reported in field studies for transgenic lines with increased RuBP regeneration grown under CO_2 enrichment[5,26], highlight the potential of manipulating electron transport and RUBP regeneration in the development of new varieties able to sustain photosynthesis and yields under climate change scenarios.

MATERIALS AND METHODS

Generation of constructs and transgenic plants

Constructs were generated using Golden Gate cloning[39,40] or Gateway cloning technology[41]. Transgenes were under the control of CaMV35S and FMV constitutive promoters. Construct detail below and in **Supplementary Fig. 6**.

For *N. tabacum* cv. Petit Havana, the codon optimised cyanobacterial bifunctional fructose-1,6-bisphosphatases/sedoheptulose-1,7-bisphosphatase (FBP/SBPase; *slr2094* Synechocystis sp. PCC 7942 [2] linked to the geraniol synthase transit peptide [42] and the codon optimised *P. umbilicalis's* cytochrome c_6 (AFC39870) with the chlorophyll a-b binding protein 6 transit peptide from Arabidopsis (AT3G54890) were used to generate Golden Gate[40] over-expression constructs (EC23083 and EC23028) driven by the FMV [43] and CaMV 35S promoters respectively (**Supplementary Fig. 6A**).

For *N. tabacum* cv. Samsun, the full-length *P. umbilicalis* cytochrome c_6 linked to the transit peptide from the light-harvesting complex I chlorophyll a/b binding protein 6 (At3g54890) were used to generate an over-expression construct driven by the CaMV 35S promoter; B2-C6 in the vector pGWB2[41] (**Supplementary Fig. 6B**). The recombinant plasmid B2-C6, was introduced into SBPase over-expressing tobacco cv. Samsun[3] using *Agrobacterium tumefaciens* AGL1 via leaf-disc transformation[44]. Primary transformants (39) (T0 generation) were regenerated on MS medium containing kanamycin (100mg L^{-1}), hygromycin (30 mg L^{-1}) and augmentin (500 mg L^{-1}). Plants expressing the integrated transgenes were screened using RT-PCR (data not shown).

In a similar fashion, the recombinant plasmids EC23083, and EC23028 were introduced into wild type tobacco (*Nicotiana tabacum*) cv Petit Havana, using *A. tumefaciens* strain LBA4404 via leaf-disc transformation[44], and shoots were regenerated on MS medium containing, hygromycin (20 mg L^{-1}) and cefotaxime (400 mg L^{-1}). Hygromycin resistant primary transformants (T0 generation) with established root systems were transferred to soil and allowed to self-fertilize.

Between twelve and 60 independent lines were generated per construct and 3-4 lines were taken forward for full analysis. Control (CN) plants used in this study were a

combined group of WT and null segregants from the transgenic lines, verified by PCR for non-integration of the transgene.

Plant Growth

Controlled conditions

Wild-type tobacco plants and T1 progeny resulting from self-fertilization of transgenic plants were grown to seed in soil (Levington F2, Fisons, Ipswich, UK). Lines of interest were identified by immunoblot and qPCR. For the experiments in the Samsun cv. the null segregants were selected from transformed lines. For Petit Havana, the null segregants were selected from the S_BC_6 lines. For experimental study, T2-T4 and F1-F3 progeny seeds were germinated on soil in controlled environment chambers at an irradiance of 130 μmol photons m^{-2} s^{-1}, 22°C, relative humidity of 60%, in a 16-h photoperiod. Plants were transferred to individual 8 cm pots and grown for two weeks at 130 μmol photons m^{-2} s^{-1}, 22°C, relative humidity of 60%, in a 16-h photoperiod. Plants were transferred to 4 L pots and cultivated in a controlled environment glasshouse (16-h photoperiod, 25°C-30°C day/20°C night, and natural light supplemented under low light induced by cloud cover with high-pressure sodium light bulbs, giving 380-1000 μmol m^{-2} s^{-1} (high-light) from the pot level to the top of the plant, respectively). Positions of the plants were changed 3 times a week and watered regularly with a nutrient medium[45]. Plants were positioned such that at maturity, a near-to-closed canopy was achieved and the temperature range was maintained similar to the ambient external environment. Four leaf discs (0.8-cm diameter) were taken for immunoblot analysis and FBPase activity. These disks were taken from the same areas of the leaf used for photosynthetic measurements, immediately plunged into liquid N_2 and stored at -80°C.

Field

Plants were grown as described in Lopez-Calcagno et al[36]. The field site was situated at the University of Illinois Energy Farm (40.11°N, 88.21°W, Urbana, IL). Two different experimental designs were used in 2 different years.

2016: Replicated control design (**Supplementary Fig. 7A**). Plants were grown in rows, spaced 30 cm apart with the outer boundary being a wild-type border. The entire experiment was surrounded by two rows of wild-type borders. Plants were irrigated when required using rain towers. T2 seed was germinated and after 11 d were moved to individual pots (350 mL). The seedlings were grown in the glasshouse for further 15 d before being moved into the field, and allowed to grow in the field for 14 d before harvest.

2017: Two experiments were carried out two weeks apart. A blocks-within-rows design was used (**Supplementary Fig. 7B**) where 1 block holds one line of each of the five manipulations and each row has all lines. The central 20 plants of each block are divided into five rows of four plants per genotype. The 2017 Exp.1 contained controls (WT and null segregants), FBP/SBPase expressing lines (S_B) and cytochrome c_6 expressing lines (C_6). The 2017 Exp. 2 contained controls (WT and null segregants), cytochrome c_6 expressing lines (C_6), and FBP/SBPase + cytochrome c_6 expressing lines (S_BC_6). Seed was germinated and after 12 d moved to hydroponic trays (Trans-plant Tray GP009 6912 cells; Speedling Inc., Ruskin, FL), and grown in the glasshouse for 20 d before being moved to the field. The plants were allowed to grow in the field until flowering (approximately 30 d) before harvest.

The field was prepared in a similar fashion each year as described in Kromdijk et al.[46]. Light intensity (LI-quantum sensor; LI-COR) and air temperature (Model 109 temperature probe; Campbell ScientificInc, Logan, UT) were measured nearby on the same field site, and half-hourly averages were logged using a data logger (CR1000; Campbell Scientific).

cDNA generation and RT-PCR

Total RNA was extracted from tobacco leaf disks (sampled from glasshouse grown plants and quickly frozen in liquid nitrogen) using the NucleoSpin® RNA Plant Kit (Macherey-Nagel, Fisher Scientific, UK). cDNA was synthesized using 1 µg total RNA in 20 µl using the oligo-dT primer according to the protocol in the RevertAid Reverse Transcriptase kit (Fermentas, Life Sciences, UK). cDNA was diluted 1 in 4 to a final concentration of 12.5ng μL^{-1}. For semi quantitative RT-PCR, 2 µL of RT reaction mixture (100 ng of RNA) in a total volume of 25 µL was used with DreamTaq DNA Polymerase (Thermo Fisher Scientific, UK) according to

manufacturer's recommendations. PCR products were fractionated on 1.0% agarose gels. For qPCR, the SensiFAST SYBR No-ROX Kit was used according to manufacturer's recommendations (Bioline Reagents Ltd., London, UK). Primers used for semi quantitative RT-PCR can be seen in **supplementary Table 1.**

Protein Extraction and immunoblot analysis

Leaf discs sampled as described above were ground in dry ice and protein extractions performed as described in Lopez-Calcagno et al.[47], or using the nucleospin RNA/Protein kit (Macherey-Nagel (http://www.mn-net.com/) during RNA preparations. Protein quantification was performed using the protein quantification Kit from Macherey-Nagel. Samples were loaded on an equal protein basis, separated using 12% (w/v) SDS-PAGE, transferred to a nitrocellulose membrane (GE Healthcare Life science, Germany), and probed using antibodies raised against SBPase and FBP/SBPase. Proteins were detected using horseradish peroxidase conjugated to the secondary antibody and ECL chemiluminescence detection reagent (Amersham, Buckinghamshire, UK). SBPase antibodies are previously characterised[3,48]. FBP/SBPase antibodies were raised against a peptide from a conserved region of the protein [C]-DRPRHKELIQEIRNAG-amide, and cytochrome c_6 antibodies were raised against peptide [C]-[Nle]-PDKTLKKDVLEANS-amide (Cambridge Research Biochemicals, Cleveland, UK). In addition to the aforementioned antibodies, samples were probed using antibodies raised against transketolase[49,50] and the Glycine decarboxylase H-protein as loading controls. Glycine decarboxylase H-protein antibodies were previously characterised in Timm et al.[51].

Protein Extraction for cytochrome c_6

Whole leaves were harvested from 8 week old plants, washed in cold water and then wiped with a cloth soaked in 80 % ethanol to remove the majority of leaf residue. The leaves were then washed twice more in cold water, the mid rib was removed and 50 g of the remaining tissue was placed in a sealed plastic bag and stored overnight in the dark at 4˚C. Proteins were extracted as in Hiyama[52], with a few modifications. Leaf tissue was homogenised in 250 ml of chilled chloroplast preparation buffer (50 mM sodium

phosphate buffer, pH 7, 10 mM NaCl) for 30 seconds. The solution was then filtered through 4 layers of muslin cloth and centrifuged at 10,000 g for 5 minutes. The resulting pellet was then gently resuspended in 50 ml of chilled chloroplast preparation buffer and the chlorophyll concentration was measured and adjusted to approximately 2 mg ml^{-1}. The resultant mixture was then added to two volumes of preheated (45˚C) solubilisation medium (50 mM Tris-HCl pH 8.8 and 3% triton X) and incubated at 45˚C for 30 minutes and then chilled in an ice bath for a further 30 minutes before centrifugation at 12000 g for 30 minutes. The supernatant was stored at -80˚C for use in the next stage. To purify cytochrome c_6 protein a Biorad Econo-Pac High-Q, 5 ml type wash column was used at a flow rate of 1ml min-1. First the column was prepared by washing it with 100 ml of starting buffer (Starting buffer: 10 mM Tris-HCl pH 8.8, 0.2% triton X 100 and 20% sucrose). Then the protein mixture from the previous step was diluted with an equal volume of chilled starting buffer and passed through the column at a flow rate of 1 ml min^{-1}. Once all the protein was loaded onto the column it was then washed with 1000 ml of starting buffer supplemented with 10 mM NaCl. Then 300 ml of starting buffer supplemented with 50 ml NaCl and finally a linear gradient of starting buffer from 50 to 200 mM NaCl over a period of 4 hours at 1 ml min^{-1} was performed and aliquots were collected. Samples were mixed with loading buffer (50% glycerol, 25% β-mecaptoethanol, 25% EDTA (300μl)) and loaded on an equal protein basis, separated using 18% (w/v) SDS-PAGE, transferred to nitrocellulose membrane, and probed using antibodies raised against cytochrome c_6.

Determination of FBPase Activity by Phosphate Release

FBPase activity was determined by phosphate release as described previously for SBPase with minor modifications[6]. Leaf discs were isolated from the same leaves and frozen in liquid nitrogen after photosynthesis measurements were completed. Leaf discs were ground to a fine powder in liquid nitrogen and immersed in extraction buffer (50 mM HEPES, pH8.2; 5 mM MgCl; 1 mM EDTA; 1 mM EGTA; 10% glycerol; 0.1% Triton X-100; 2 mM benzamidine; 2 mM aminocapronic acid; 0.5 mM phenylmethylsulfonylfluoride; 10 mM dithiothreitol), centrifuged 1 min at 14,000 xg, 4°C. The resulting supernatant (1 ml) was desalted through an NAP-10 column (Amersham) and stored in liquid nitrogen. The assay was carried out as descried in Simkin et al.[6]. In brief, 20 μl of extract was added to

80 µl of assay buffer (50 mM Tris, pH 8.2; 15 mM $MgCl_2$; 1.5 mM EDTA; 10 mM DTT; 7.5 mM fructose-1,6-bisphosphate) and incubated at 25 °C for 30 min. The reaction was stopped by the addition of 50 µl of 1 M perchloric acid. 30 µl of samples or standards (PO^{3-}_4 0.125 to 4 nmol) were incubated 30 min at room temperature following the addition of 300 µl of Biomol Green (Affiniti Research Products, Exeter, UK) and the A620 was measured using a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA). Activities were normalized to transketolase activity[53].

Chlorophyll fluorescence imaging screening in seedlings

Chlorophyll fluorescence imaging was performed on 2-3 week-old tobacco seedlings grown in a controlled environment chamber at 130 µmol mol^{-2} s^{-1} and ambient (400 µmol mol^{-1}) CO_2. Chlorophyll fluorescence parameters were obtained using a chlorophyll fluorescence (CF) imaging system (Technologica, Colchester, UK[54,55]). The operating efficiency of photosystem two (PSII) photochemistry, F_q'/F_m', was calculated from measurements of steady state fluorescence in the light (F') and maximum fluorescence (F_m') following a saturating 800 ms pulse of 6300 µmol m^{-2} s^{-1} PPFD and using the following equation $F_q'/F_m' = (F_m'-F')/F_m'$. Images of F_q'/F_m' were taken under stable PPFD of 600 µmol m^{-2} s^{-1} for Petite Havana and 650 µmol m^{-2} s^{-1} for Samsun[56-58].

Leaf Gas Exchange

Photosynthetic gas-exchange and chlorophyll fluorescence parameters were recorded using a portable infrared gas analyser (LI-COR 6400; LI-COR, Lincoln, NE, USA) with a 6400-40 fluorometer head unit. Unless stated otherwise, all measurements were taken with LI-COR 6400 cuvette. For plants grown in the glasshouse conditions were maintained at a CO_2 concentration, leaf temperature and vapour pressure deficit (VPD) of 400 µmol mol^{-1}, 25 °C and 1 ± 0.2 kPa respectively. The chamber conditions for plants grown under field conditions had a CO_2 concentration of 400 µmol mol^{-1}, block temperature was set to 2 °C above ambient temperature (ambient air temperature was measure before each curve) and VPD was maintained as close to 1 kPa as feasible possible.

A/C_i response curves (Photosynthetic capcacity)

The response of net photosynthesis (*A*) to intracellular CO_2 concentration (C_i) was measured at a saturating light intensity of 2000 µmol mol^{-2} s^{-1}. Illumination was provided by a red-blue light source attached to the leaf cuvette. Measurements of *A* were started at ambient CO_2 concentration (C_a) of 400 µmol mol^{-1}, before C_a was decreased step-wise to a lowest concentration of 50 µmol mol^{-1} and then increased step-wise to an upper concentration of 2000 µmol mol^{-1}. To calculate the maximum saturated CO_2 assimilation rate (A_{max}), maximum carboxylation rate (Vc_{max}) and maximum electron transport flow (J_{max}), the C3 photosynthesis model[59] was fitted to the A/C_i data using a spreadsheet provided by Sharkey et al.[60]. Additionally, chlorophyll fluorescence parameters including PSII operating efficiency (F_q'/F_m') and the coefficient of photochemical quenching (q_P), mathematically identical to the PSII efficiency factor (F_q'/F_v') were recorded at each point.

A/*Q* response curves

Photosynthesis as a function of light (*A*/*Q* response curves) was measured under the same cuvette conditions as the A/C_i curves mentioned above. Leaves were initially stabilized at saturating irradiance of 2200 to µmol m^{-2} s^{-1}, after which *A* and g_s were measured at the following light levels: 2000, 1650, 1300, 1000, 750, 500, 400, 300, 200, 150, 100, 50 and 0 µmol m^{-2} s^{-1}). Measurements were recorded after *A* reached a new steady state (1-3 min) and before g_s changed to the new light levels. Values of *A* and g_s were used to estimate intrinsic water-use efficiency ($iWUE = A/g_s$)

Statistical Analysis

All statistical analyses were done using Sys-stat, University of Essex, UK, and R (https://www.r-project.org/). For harvest data, seedling chlorophyll imaging and enzyme activities, analysis of variance and Post hoc Tukey test were done. For gas exchange curves, data were compared by linear mixed model analysis using lmer function and type

III anova[61]. Significant differences between manipulations were identified using contrasts analysis (lsmeans package).

Figure Legends

Fig 1. Screening of transgenic plants overexpressing FBP/SBPase, SBPase, and cytochrome c_6.

(**a**) Transcript levels in S_B, C_6, S_BC_6, S and SC_6 lines compared to controls (CN). (**b**) FBPase activity in S_B and BC_6 lines relative to controls. (**c-d**) Chlorophyll fluorescence imaging of plants grown in controlled environmental conditions used to determine F_q'/F_m' (maximum PSII operating efficiency) at 600-650 µmol m^{-2} s^{-1}. n=7-10. * indicates lines which are statistically different to control groups ($p<0.05$).

Fig 2. Photosynthetic responses of transgenic plants grown in glasshouse.

Photosynthetic carbon fixation rates, actual operating efficiency of PSII in the light (F_q'/F_m'), electron sinks pulling away from PSII (F_q'/F_v') and PSII maximum efficiency (F_v'/F_m') are presented in (**a**) mature leaves of cv. Petit Havana and (**b**) mature and (**c**) developing leaves of cv. Samsun. Parameters were determined as a function of increasing CO_2 concentrations at saturating-light levels in developing (11-13cm in length) and mature leaves from control and transgenic plants. Plants were grown in natural light conditions in the glasshouse where light levels oscillated between 400 and 1000 µmol m^{-2} s^{-1} (supplemental light maintain a minimum of 400 µmol m^{-2} s^{-1}). Lines expressing FBP/SBPase (S_B), Cytochrome c_6 (C_6), FBP/SBPase and Cytochrome c_6, (S_BC_6) SBPase (S) and SBPase and Cytochrome c_6 (SC_6). Control group (CN) represent both WT and azygous plants. Evaluations are based on 3-4 plants individual plants per line, and 3 to 4 independent transgenic lines per manipulation. Asterisks indicate significance between transgenics and control group, using a linear mixed-effects model and type III ANOVA and contrast analysis, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Fig 3. Increased SBPase or expression of FBP/SBPase and cytochrome c_6 increases biomass in glasshouse grown plants.

Tobacco plants were germinated in growth cabinets and moved to the glasshouse at 10-14 d post-germination forty-day-old (cv. Petit Havana) or fifty-six-day-old (cv. Samsun)

plants were harvested and plant height, leaf area and above-ground biomass (dry weight) were determined. Control group represent both WT and azygous plants. Mean and SE presented. n= 5-6 individual plants from 2 to 4 independent transgenic lines. Asterisks indicate significance between transgenics and control group, or between genotypes using ANOVA with Tukey's post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001.

Fig 4. Simultaneous expression of FBP/SBPase and cytochrome *c*$_6$ increases biomass in field grown plants.

(**a**) **F**orty-day-old (young) 2016 field-grown plants (plants were germinated and grown in glasshouse conditions for 26 d and then allowed to grow in the field in summer 2016 for 14 d); (**b-c**) Fifty-seven-day-old or sixty-one-day-old (flowering) 2017 field-grown plants (plants were germinated and grown in glasshouse conditions for 26 d and then allowed to grow in the field in summer 2017 until when flowering established, circa 30 d). Light grey bars represent FBP/SBPase expressing plants (S_B), dark grey bars represent cytochrome *c*$_6$ expressing plants (C_6) and white bars represent plant expressing both transgenes (S_BC_6). Plant height, leaf area and total above-ground biomass (dry weight) are displayed. Control group represent both WT and azygous plants. Mean ± SE presented. 2-3 independent lines per manipulation 6 (**a**) or 24 (**b-c**) plants per line. Asterisks indicate significance between transgenics and control group, or between transgenic groups, *p < 0.05, **p < 0.01, ***p < 0.001.

Fig 5. Photosynthetic capacity of field-grown transgenic plants.

Photosynthetic carbon fixation rates and operating efficiency of PSII as a function of increasing CO_2 concentrations at saturating-light levels in mature leaves from CN and transgenic plants. (**a**) 2017 experiment 1: Lines expressing FBP/SBPase (S_B) and cytochrome *c*$_6$ (C_6). (**b**) 2017 experiment 2: Lines expressing cytochrome *c*$_6$ (C_6) and FBP/SBPase and cytochrome *c*$_6$ (S_BC_6). Control group (CN) represent both WT and azygous plants. Evaluations are based on 4-5 individual plants from 2-3 independent transgenic lines. Asterisks indicate significance between transgenics and control group, using a linear mixed-effects model and type III ANOVA, *p < 0.05.

Fig 6. Simultaneous expression of FBP/SBPase and cytochrome c_6 can increase water use efficiency under field conditions.

(**a**) Net CO_2 assimilation rate (A), (**b**) Stomatal conductance (g_s), (**c**) Intercellular CO_2 concentration (C_i), and (**d**) Intrinsic water-use efficiency (i*WUE*) as a function of light (PPFD) in field-grown plants. Lines expressing cytochrome c_6 (C_6) and FBP/SBPase and cytochrome c_6 (S_BC_6). Control group (CN) represent both WT and azygous plants. Mean ± SE presented. Evaluations are based on 4-5 individual plants from 2-3 independent transgenic lines per manipulation. Asterisks indicate significance between transgenics and control group, using a linear mixed-effects model and type III ANOVA and contrast analysis, *p < 0.05, **p < 0.01, ***p < 0.001.

Table 1. Maximum electron transport rate and RuBP regeneration (J_{max}), carboxylation rate of Rubisco (Vc_{max}) and maximum assimilation (A_{max}) of WT and transgenic lines. Results were determined from the A/C_i curves in Figure 2 using the equations published by von Caemmerer and Farquhar[62]. Statistical differences are shown in boldface (* p<0.05). Mean and SE are shown.

			A/C_i		
	Leaf Stage	Line	Vc_{max} (μmol m^{-2} s^{-1})	J_{max} (μmol m^{-2} s^{-1})	A_{max} (μmol m^{-2} s^{-1})
Samsun	Developing	CN	74.2 ± 5.1	159.1 ± 5.3	30.1 ± 1.01
		S	83.9 ± 4.4	**173.9 ± 5.5***	**33.5 ± 0.87***
		SC_6	86.5 ± 3.5	**181.2 ± 3.6***	**33.6 ± 1.13***
	Mature	CN	77.3 ± 3.3	171.0 ± 6.0	31.6 ± 0.98
		S	80.8 ± 5.4	184.8 ± 1.3	32.7 ± 0.82
		SC_6	**90.3 ± 3.3***	**193.1 ± 5.4***	**34.5 ± 1.06***
	Mature	CN	69.6 ± 2.0	121.5 ± 1.3	24.6 ± 0.47

	S_B	69.0 ± 5.1	128.7 ± 3.8	27.0 ± 0.82
Petit Havana	C_6	79.3 ± 7.0	129.9 ± 5.1	25.6 ± 0.53
	S_BC_6	76.5 ± 4.2	132.0 ± 3.8	**27.4 ± 0.75***

Supplementary Information

Supplementary Figure 1. Biochemical Analysis of the transgenic Petit Havana plants expressing cyFBP/SBPase and SBPase.

(**a**) Immunoblot analysis of protein extracts from mature leaves of evaluated S_B and S_BC_6 lines compared to control, blotted against cyFBP/SBPase antibody. (**b**) Immunoblot analysis of protein extracts from mature leaves of evaluated S and SC_6 lines compared to controls, blotted against SBPase antibody. Expression of H-protein from the glycine cleavage system, Transketolase (TK) and Rubisco were used as loading controls.

Supplementary Figure 2. Complete data set for enzyme assays in plants analysed. FBPase enzyme activities from cv. Petite Havana plants. Bars represent activities from individual plants from studied lines.

Supplementary Figure 3. Biochemical analysis of the transgenic *N. tabacum* cv. Petit Havana plants expressing cytochrome c_6. (a) Immunoblot analysis of protein extract from a pool of developing leaves of evaluated C_6 lines compared to WT and null segregant (A) controls and to a *Porphyra umbilicalis* protein extract (P). (**b)** Ponceau staining of membrane to show similar loading of plant leaf extracts.

Supplementary Figure 4. Increased SBPase or expression of FBP/SBPase and cytochrome c_6 increases biomass in GH grown plants. Additional parameters.

Tobacco plants were germinated in growth cabinets and moved to the glasshouse at 10-14 d post-germination. Forty-day-old (cv. Petit Havana) or fifty-six-day-old (cv. Samsun) plants were harvested and leaf number, leaf dry weight and stem dry weight were determined. Control group represent both WT and azygous plants. Mean and SE presented. n= 5-6 individual plants from 2 to 4 independent transgenic lines. Asterisks

indicate significance between transgenics and control group, or between genotypes using ANOVA with Tukey's post hoc test, *p < 0.05, **p < 0.01, ***p < 0.001.

Supplementary Figure 5. Response of gas exchange parameters to absorbed light intensity in field-grown *N. tabacum* plants expressing cyFBP/SBPase or cytochrome *c*$_6$.

(**a**) Net CO_2 assimilation rate (A), (**b**) Stomatal conductance (g_s), (**c**) Intercellular CO_2 concentration (C_i), and (**d**) Intrinsic water-use efficiency (*iWUE*) as a function of absorbed light intensity (PPFD) in field-grown plants. Single manipulation 2017 experiment 1, Mean +/- SE presented. Lines expressing FBP/SBPase (S_B) and cytochrome c_6 (C_6). Control group (CN) represent both WT and azygous plants. Evaluations are based on 4-5 individual plants from 2-3 independent transgenic lines per manipulation. Asterisks indicate significance between groups, using a linear mixed-effects model and type III ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001.

Supplementary Figure 6. Schematic representation of the constructs used.

(**a**) FBP/SBPase (EC23083) and cytochrome c_6 constructs for expression in *N. tobacum* cv. Petit Havana and (**b**) cytochrome c_6 (B2-C6) construct for expression in cv. Samsun. RB, T-DNA right border; NosP, nopaline synthase promoter; NTP II, neomycin phosphotransferase gene; Tnos, nopaline synthase terminator; 35S, cauliflower mosaic virus 35S promoter, FMV, figwart mosaic virus promoter. Constructs were used to transform wild type tobacco or Samsun tobacco over-expressing SBPase[3,6].

Supplementary Figure 7. Field experiments layouts

Schematic to represent the experimental field designs for 2016 and both 2017 experiments. (**a**) 2016 Field experiment were designed to a replicated control design. Each row was 4x14 with the outer plant being a wild type boarder control. Two lines per row were planted in an alternating pattern. The controls for each transgenic construct

were the two rows of controls either side of the transgenic row. (**b**) The two 2017 field design was randomised rows split into three blocks, where one row has all 15 lines and one block had one line of each construct. Rows were randomised using RAND function, Microsoft Excel 2010. Both experiments were surrounded by a wild type border and each separate experiment was bordered by two lines of wild type.

Supplementary Table 1; Primers used for semi-quantitative RT-PCR.

Cultivar	gene	Forward Primer	Reverse Primer	Amplicon
Samsun cv	Cytochrome c_6	5'TGCTGCAGATCTAGATAATGG'3	5'CGATCGTTCAAACATTTGGCA'3	354 bp
	SBPase	5'ATGGAGACCAGCATCGCGTGCTACTC'3	5'CGATCGTTCAAACATTTGGCA'3	1269 bp
	EF	5'TGAGATGCACCACGAAGCTC'3	5'CCAACATTGTCACCAGGAAGTG'3	479 bp
Petite havana	Cytochrome c_6	5'TCGCTTATGAGCTGTGGCAT'3	5'CAACTAGCCGACCACCGAAG'3	652 bp
	FBP/SBPase	5'TGCTTCTGCTAAGTGGATGGG'3	5'ACATCTCATAGCAGCAGCAGA'3	427 bp
	EF	5'TGAGATGCACCACGAAGCTC'3	5'CCAACATTGTCACCAGGAAGTG'3	479 bp

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Author contributions

P.E.L.C and A.J.S. generated transgenic plants. P.E.L.C, A.J.S, K.L.B. and S.J.F. performed molecular and biochemical experiments. P.E.L.C, A.J.S and K.L.B carried out plant phenotypic and growth analysis and performed gas exchange measurement. A.J.S and S.J.F performed enzyme assays on selected lines; all authors carried out data analysis on their respective contributions; C.A.R and T.L designed and supervised the research; P.E.L.C., A.J.S and C.A.R wrote the manuscript, P.E.L.C, K.L.B. and A.J.S contributed equally to the completion of this work.

Competing interests: The authors declare no competing financial interests

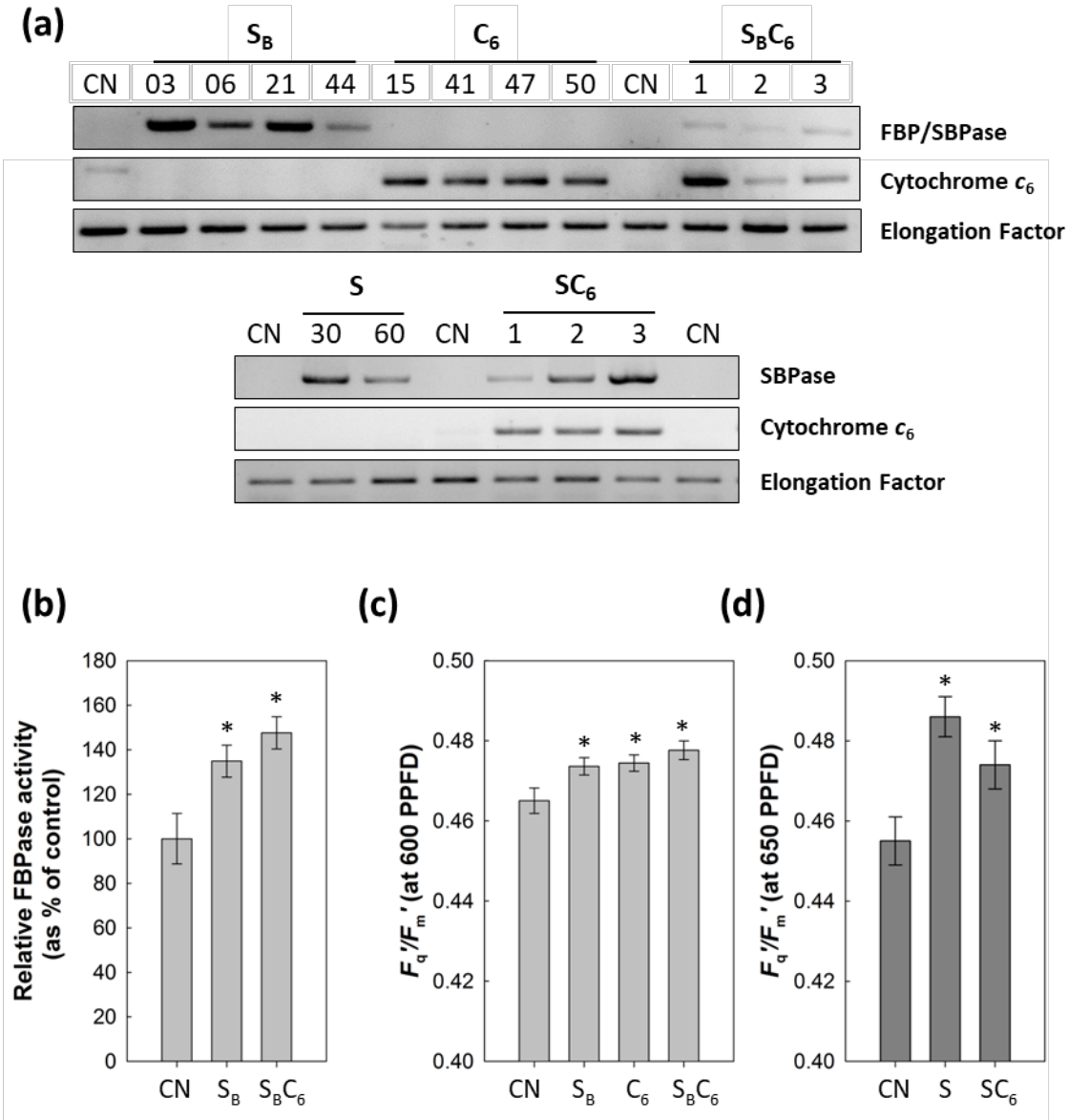

Fig. 1. Screening of transgenic plants overexpressing cyFBP/SBPase, SBPase, and cytochrome c_6.

(**a**) Transcript levels in S_B , C_6, S_BC_6, S and SC_6 lines compared to controls (CN). (**b**) FBPase activity in S_B and S_BC_6 lines relative to controls. (**c-d**) Chlorophyll fluorescence imaging of control, S_B , C_6, S_BC_6, S and SC_6 plants grown in controlled environmental conditions used to determine F_q'/F_m' (maximum PSII operating efficiency) at 600-650 µmol m^{-2} s^{-1}. n=7-10 *indicates lines which are statistically different to control groups ($p<0.05$).

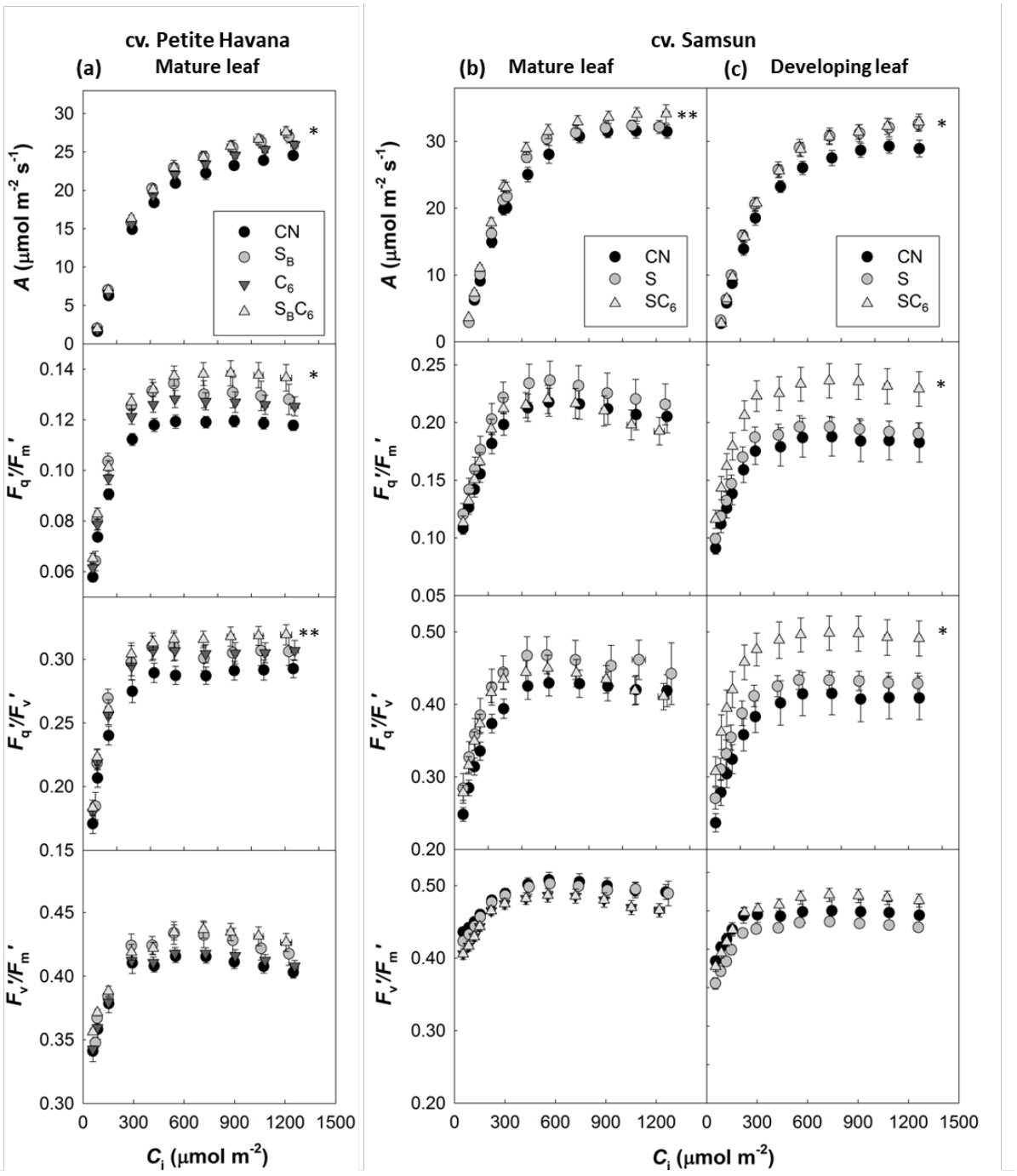

Fig 2. Photosynthetic responses of transgenic plants grown in glasshouse.

Photosynthetic carbon fixation rates, actual operating efficiency of PSII in the light (F_q'/F_m'), electron sinks pulling away from PSII (F_q'/F_v') and PSII maximum efficiency (F_v'/F_m') are presented in (**a**) mature leaves of cv. Petit Havana and (**b**) mature and (**c**) developing leaves of cv. Samsun. Parameters were determined as a function of increasing CO_2 concentrations at saturating-light levels in developing (11-13cm in length) and mature leaves from control and transgenic plants. Plants were grown in natural light conditions in the glasshouse where light levels oscillated between 400 and 1000 µmol m^{-2} s^{-1} (supplemental light maintain a minimum of 400 µmol m^{-2} s^{-1}). Lines expressing FBP/SBPase (S_B), Cytochrome c_6 (C_6), FBP/SBPase and Cytochrome c_6, (S_BC_6) SBPase (S) and SBPase and Cytochrome c_6 (SC_6). Control group (CN) represent both WT and azygous plants. Evaluations are based on 3-4 plants individual plants per line, and 3 to 4 independent transgenic lines per manipulation. Asterisks indicate significance between transgenics and control group, using a linear mixed-effects model and type III ANOVA and contrast analysis, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

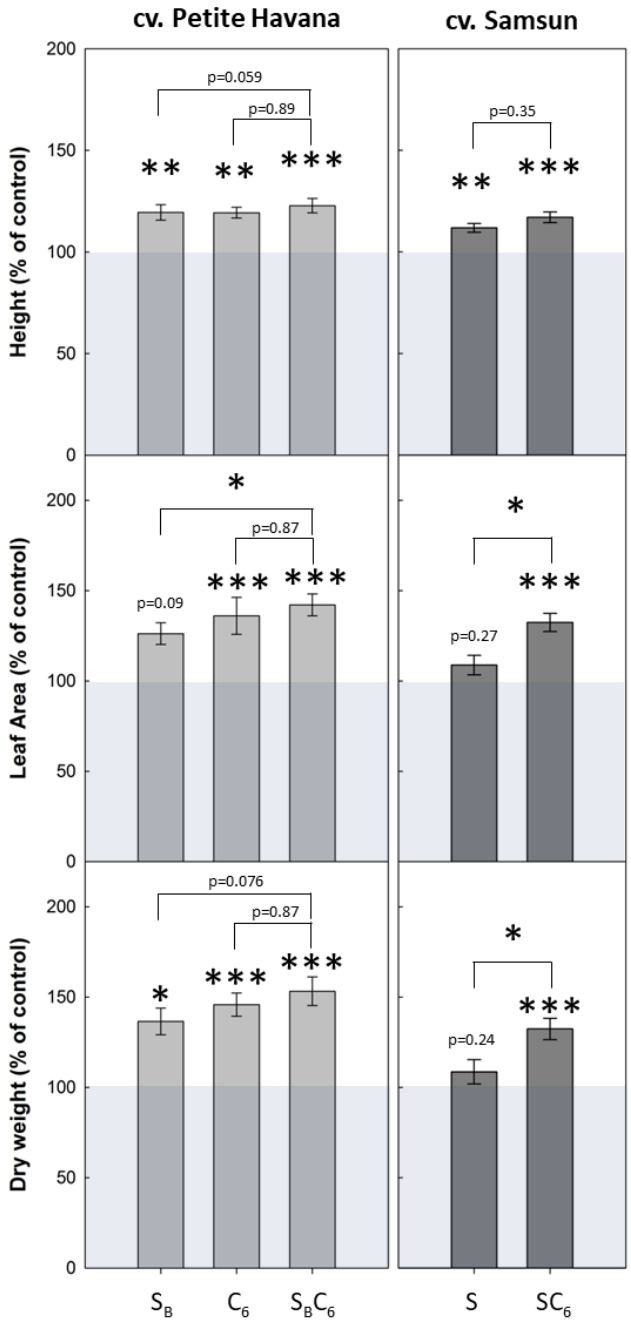

Figure 3. Increased SBPase or expression of FBP/SBPase and cytochrome c_6 increases biomass in glasshouse grown plants.

Tobacco plants were germinated in growth cabinets and moved to the glasshouse at 10-14 d post-germination forty-day-old (cv. Petit Havana) or fifty-six-day-old (cv. Samsun) plants were harvested and plant height, leaf area and above-ground biomass (dry weight) were determined. Control group represent both WT and azygous plants. Mean and SE presented. n= 5-6 individual plants from 2 to 4 independent transgenic lines. Asterisks indicate significance between transgenics and control group, or between genotypes using ANOVA with Tukey's post hoc test, *P < 0.05, **P < 0.01, ***P < 0.001.

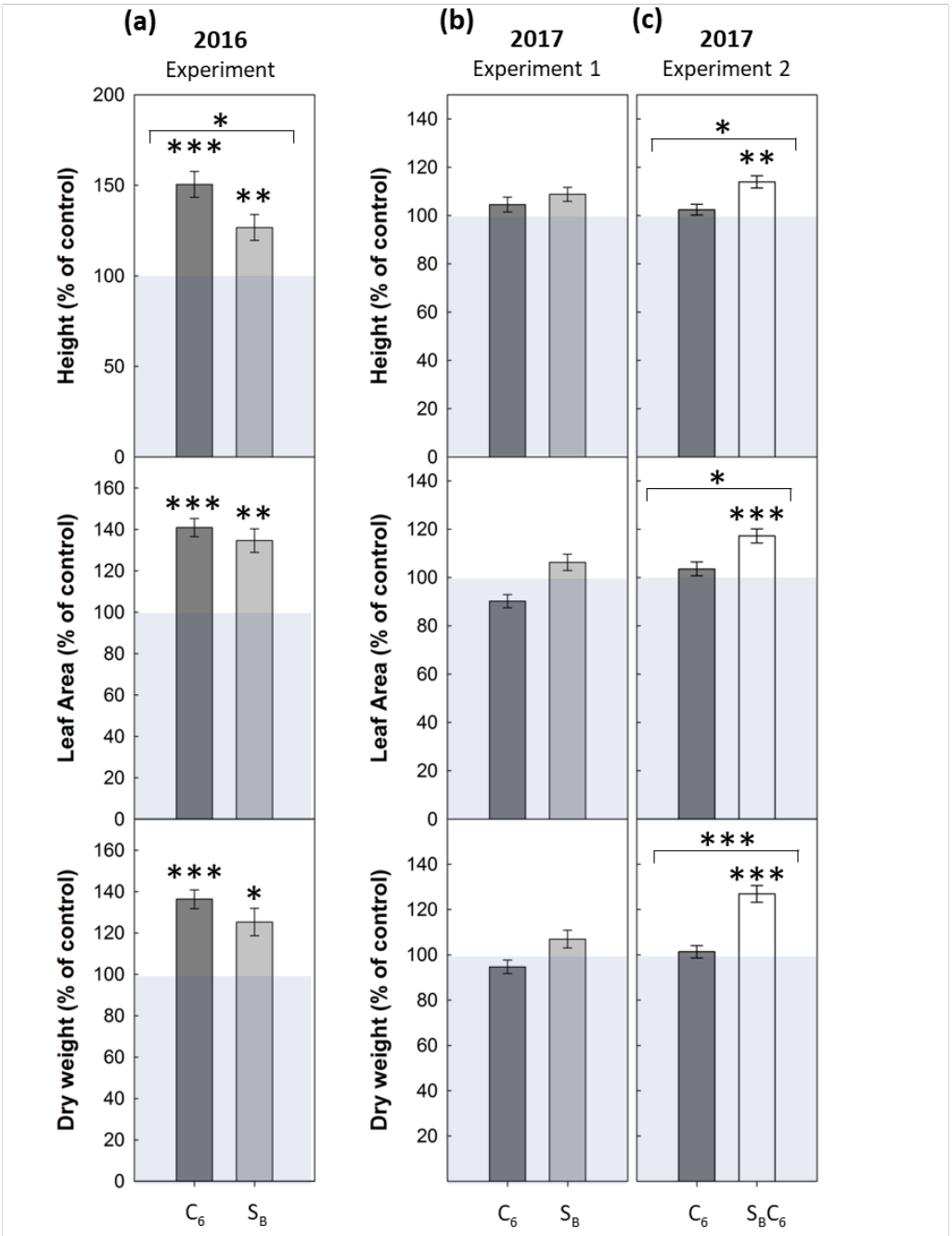

Figure 4. Simultaneous expression of FBP/SBPase and cytochrome c_6 increases biomass in field grown plants.

(**a**) forty-day-old (young) 2016 field-grown plants (plants were germinated and grown in glasshouse conditions for 26 d and then allowed to grow in the field in summer 2016 for 14 d); (**b-c**) Fifty-seven-day-old or sixty-one-day-old (flowering) 2017 field-grown plants (plants were germinated and grown in glasshouse conditions for 26 d and then allowed to grow in the field in summer 2017 until when flowering established, circa 30 d). Light grey bars represent FBP/SBPase expressing plants (S_B), dark grey bars represent cytochrome c_6 expressing plants (C_6) and white bars represent plant expressing both transgenes (S_BC_6). Plant height, leaf area and total above-ground biomass (dry weight) are displayed. Control group represent both WT and azygous plants. Mean ± SE presented. 2-3 independent lines per manipulation 6 (**a**) or 24 (**b-c**) plants per line. Asterisks indicate significance between transgenics and control group, or between transgenic groups, *p < 0.05, **p < 0.01, ***p < 0.001.

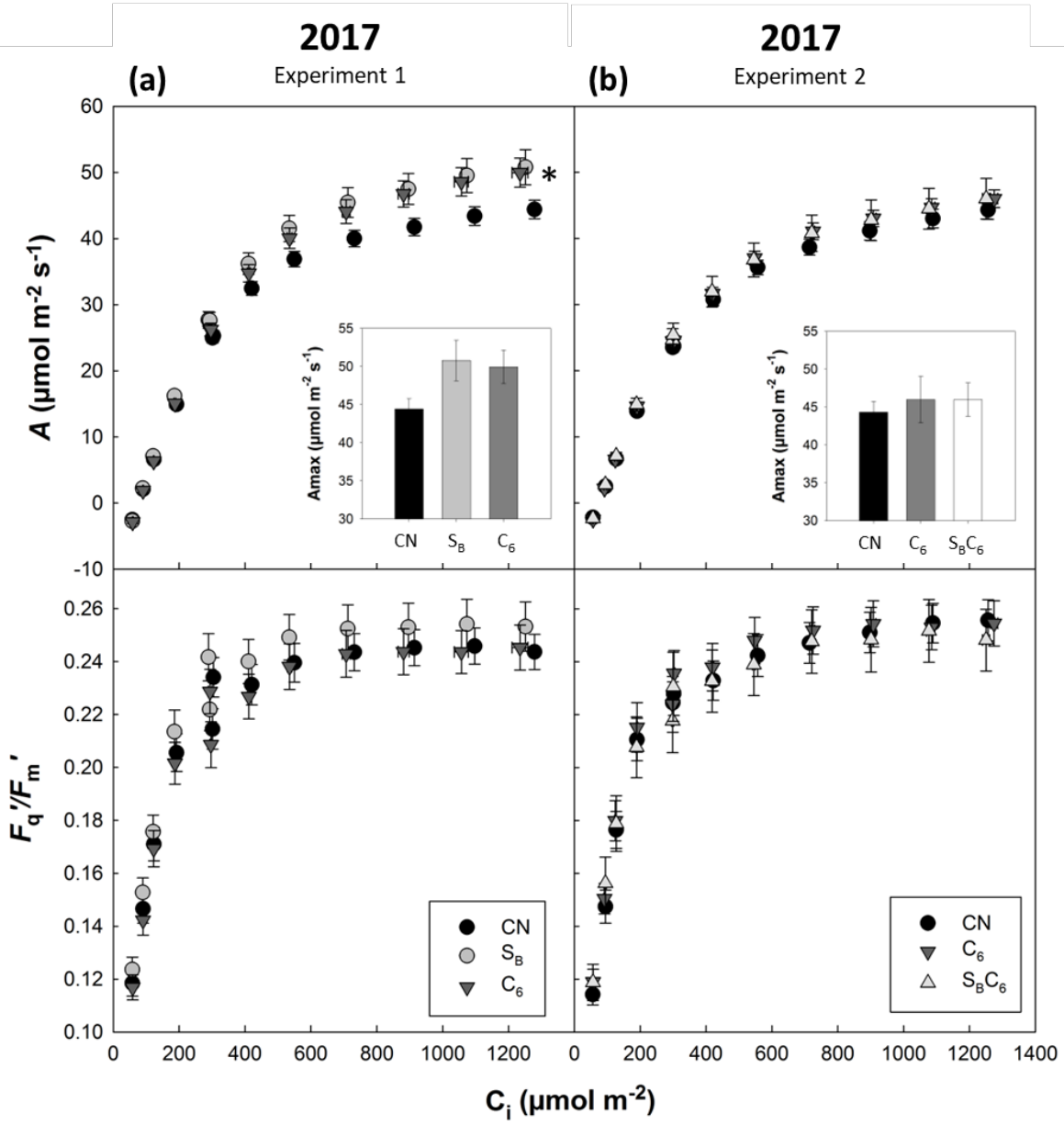

Fig 5. Photosynthetic capacity of field-grown transgenic plants.
Photosynthetic carbon fixation rates and operating efficiency of PSII as a function of increasing CO_2 concentrations at saturating-light levels in mature leaves from CN and transgenic plants. (**a**) 2017 experiment 1: Lines expressing FBP/SBPase (S_B) and cytochrome c_6 (C_6). (**b**) 2017 experiment 2: Lines expressing cytochrome c_6 (C_6) and FBP/SBPase and cytochrome c_6 (S_BC_6). Control group (CN) represent both WT and azygous plants. Evaluations are based on 4-5 individual plants from 2-3 independent transgenic lines. Asterisks indicate significance between transgenics and control group, using a linear mixed-effects model and type III ANOVA, *P < 0.05.

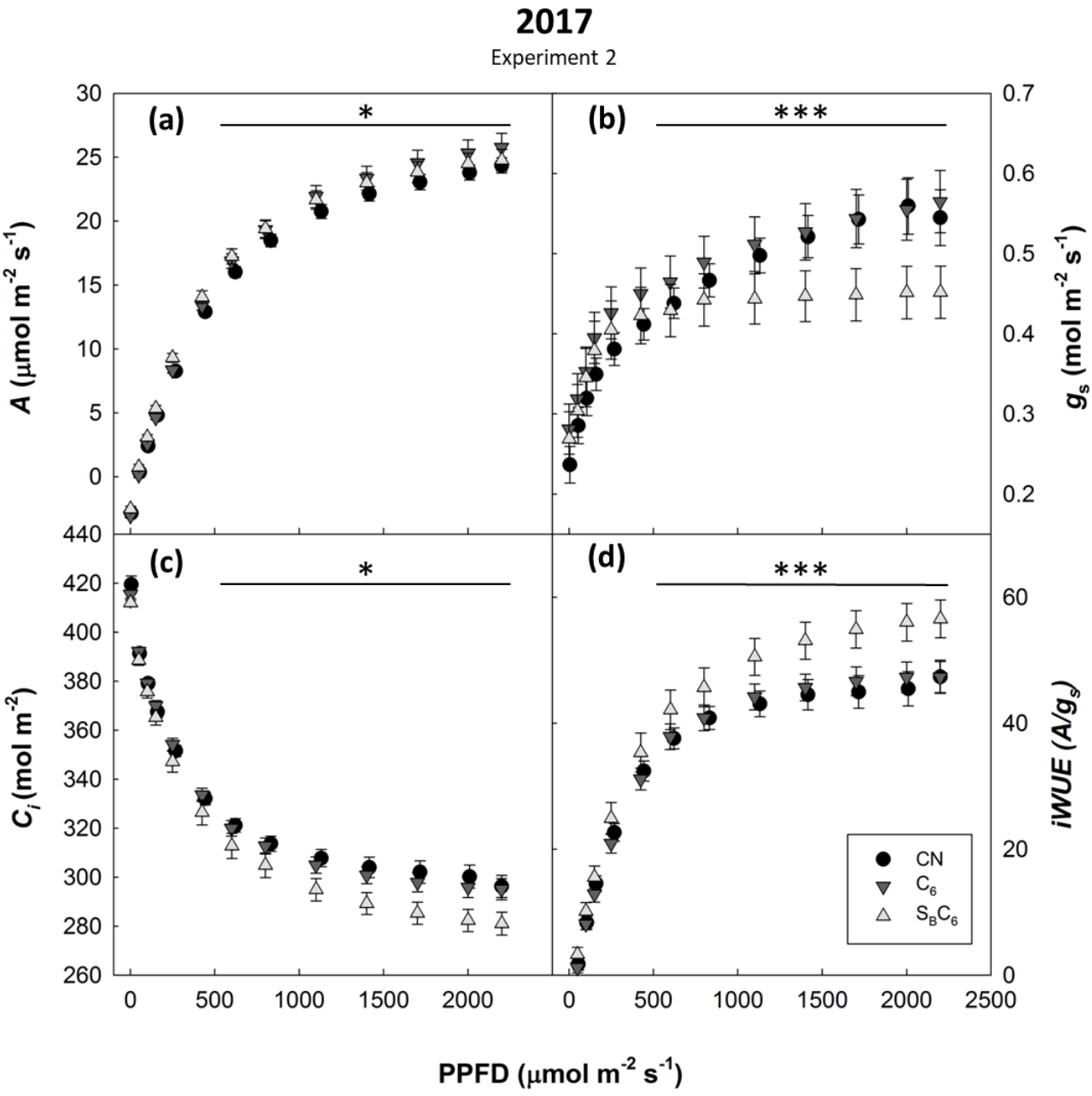

Fig 6. Simultaneous expression of FBP/SBPase and cytochrome c_6 can increase water use efficiency under field conditions.

(a) Net CO_2 assimilation rate (A), (**b**) Stomatal conductance (g_s), (**c**) Intercellular CO_2 concentration (C_i), and (**d**) Intrinsic water-use efficiency (i*WUE*) as a function of light (PPFD) in field-grown plants. Lines expressing cytochrome c_6 (C_6) and FBP/SBPase and cytochrome c_6 (S_BC_6). Control group (CN) represent both WT and azygous plants. Mean ± SE presented. Evaluations are based on 4-5 individual plants from 2-3 independent transgenic lines per manipulation. Asterisks indicate significance between groups, using a linear mixed-effects model and type III ANOVA and contrast analysis, *p < 0.05, **p < 0.01, ***p < 0.001.