Realising Increased Photosynthetic Efficiency to Increase Strawberry Yields

Nicholas Harry Doddrell

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School of Chemistry, Food and Pharmacy

Department of Food and Nutritional Sciences

Supervised by Dr Andrew Simkin, Dr Mark Else and Prof Carol Wagstaff

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Abstract

As the global population continues to rise, new solutions are required to increase productivity of food systems. One such approach is to increase the efficiency of crop photosynthesis by genetic manipulation of rate-limiting enzymes, a method proven to be successful in a range of model and cereal crop species. This thesis aims to apply this to cultivated strawberry (*Fragaria x ananassa Duch.*) by first exploring species-specific factors limiting photosynthetic efficiency and then generating transgenic lines with enhanced photosynthesis.

Variation of photosynthesis across a polytunnel was recorded to better understand photosynthesis in strawberry. It was found that availability of photosynthetically active radiation and stomatal behaviour are key factors in determining photosynthetic carbon assimilation and that this determines patterns in fruit yield seen across the tunnel.

Plasmids were constructed containing coding sequences for sedoheptulose-1,7-bisphosphatase (SBPase), a Calvin-Benson-Bassham Cycle enzyme, and adenosine diphosphate glucose pyrophosphorylase (AGPase), a starch synthesis enzyme. Both genes have formerly been implicated in limiting photosynthetic rate, making interesting targets for study. Transgenic plants expressing both genes in concert and AGPase alone were regenerated from transformed callus tissue on hygromycin selection, though this rate of regeneration was significantly delayed by the selectable marker. The photosynthetic efficiency of transgenic plants was then tested via chlorophyll fluorescence imaging. A small, non-significant increase in photosystem II operating efficiency was observed in the double expressing line only, suggesting AGPase does not limit photosynthetic rate in strawberry while SBPase might. However, both transgenic lines had an increase non-photochemical quenching, suggesting a role of starch synthesis in regulation of the photosystem.

Overall, these results lay the groundwork for realising increased photosynthetic efficiency in strawberry and other fruiting crops and describe genetic and physiological targets limiting strawberry photosynthetic rate for future study.

Thesis Structure

The thesis consists of three results chapters preceded by a review of relevant literature and general materials and methods. The results chapters are followed with general discussion, references and appendixes.

Chapter 1: Genetic approaches to increase photosynthetic efficiency and its potential application in fruiting crops

A review of the current literature in genetic improvement of photosynthesis and exploring this potential in fruiting crops by using CO_2 fertilisation as a proxy for genetically enhanced photosynthesis. This has been adapted into a review article submitted to *Plant Physiology*.

Chapter 2: Materials and Methods

A description of the materials and methods used through the thesis. Specific sections are referred to within each chapter to clarify the exact methods used for each thesis chapter.

Chapter 3: Exploring variation in photosynthetic carbon assimilation, intrinsic water use efficiency and fruit yield across the east-west axis of polytunnel grown cultivated strawberry

The first results chapter explores how photosynthesis and fruit yield varies with row positon in polytunnel grown strawberry. The chapter aims to use photosynthesis to probe why

strawberries grown in certain rows yield greater numbers and masses of fruits than others and what species-specific limitations may exist on strawberry photosynthesis.

Chapter 4: Transformation of cultivated strawberry with photosynthetic genes of interest

The second results chapter details the work undertaken to transform strawberry with the selected genes of interest, SBPase and AGPase. Work undertaken to construct plasmids is described followed by a timeline of the steps required to transform strawberry. Notable challenges, roadblocks and methods troubleshooting are also explored.

Chapter 5: Overexpression of SBPase and AGPase improves photosynthetic characteristics of cultivated strawberry individually and in concert

The final results chapter takes the plants transformed in Chapter 4 and examines their genotype and phenotype. RT-qPCR is carried out to investigate expression patterns of transformants followed by chlorophyll fluorescence imaging to understand how these transformations affected photochemistry.

Chapter 6: General discussion

A summary and discussion of the work carried out in Chapters 3 – 5 including an exploration of how these chapter relate. Challenges in this project and future work are also discussed.

Chapter 7: References

References for all chapters are listed here for continuity of the manuscript.

Chapter 8: Appendixes

Supplementary material to support the main text.

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stuff any time soon! You are my rock in this world and I could not have completed this without you.

Declaration

‘Declaration: I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.’

Signed,

Nicholas Harry Doddrell

Chapter 1 – Genetic approaches to increase photosynthetic efficiency and its potential application in fruiting crops

1.1 Introduction

1.1.1 Exposition

With the global population predicted to exceed 10 billion by the year 2055 (United Nations Department of Economic and Social Affairs Population Division, 2019), it is imperative that research is undertaken to ensure stable food security for the future. Whilst agricultural yields of key crops have risen over the last 50 years, keeping pace with demand, these increases have been exclusively brought about by advances in agricultural practices and the breeding of new varieties suited to commercial farming, such as the introduction of dwarfing genes by conventional breeding to maximise architecture and light capture and the optimisation of fertilizer regimes (Long *et al.*, 2015; Ray *et al.*, 2013). A possible alternative approach to greater yields is the improvement of crop photosynthetic efficiency. Such avenues to yield improvement deserve exploration, since to maintain adequate supplies, an increase of 70 % to 100 % in the yields of some crops (i.e. wheat, rice) will be necessary (Tilman and Clark, 2015; FAO, 2017b).

Evans and Fisher (1999) described the maximum yield attainable by a crop as the 'yield potential', characterized as the yield obtained from a crop grown under ideal conditions (i.e. grown with sufficient nutrients and water and the minimisation of external stresses, such as pests and disease). Further studies have defined the 'yield equation' as a means of identifying and quantifying the parameters that determine crop yield (Long *et al.*, 2006). The yield equation is defined as follows:

$$P_n = S_t * \varepsilon_i * \varepsilon_c / K$$

$$Y_p = \eta * P_n$$

where Y_p defines the yield potential, η defines the harvest index (i.e. biomass partitioned into the harvestable plant structures), P_n defines the primary production of biomass, S_t defines the incident solar radiation over a crop, ε_i defines the efficiency of light interception by the crop, ε_c defines the efficiency of conversion of intercepted light into biomass and K defines the energy content of the harvestable biomass (Long *et al.*, 2006). It has long been established that several of these parameters are nearing their theoretical maximum. In a review by Zhu et al., (2010), data on field grown soybean was analysed to calculate the harvest index, conversion efficiency and interception efficiency of the crop (Zhu *et al.*, 2010; Morgan *et al.*, 2005; Dermody *et al.*, 2008). Both harvest index and interception efficiency were found to be near their theoretical maxima, although large intra-crop variability in harvest index is seen in practice due to variations in growth conditions, including availability of nitrogen and time of crop sowing (Unkovich *et al.*, 2010). Conversion efficiency was ~30 % of its theoretical maximum for a field grown crop. This reveals how conventional breeding has struggled to achieve the theoretical maximum conversion efficiency resulting in carbon assimilation as a major limitation on crop yield. Conversion efficiency is limited first by the photosystem's capacity to utilise available photosynthetically active radiation (PAR), due to thermodynamic constraints and loss of excess energy as heat and light. Energy is also lost following excitation of the chlorophyll reaction centre, due to the energetic costs of carbohydrate biosynthesis, respiration to generate ATP for photosynthesis and, in C3 crops, photorespiration to minimise photodamage (Zhu *et al.*, 2010). These limitations are also present in glasshouse grown crops, with previous work suggesting that atmospheric [CO_2] is a greater driver of differences in

photosynthetic carbon assimilation than growth in field or chamber (Wilson *et al.*, 1992).

The aim of increasing crop yield is complicated further by rising global temperatures with an expected mean global temperature increase of 2 °C by 2050, a concomitant increase in atmospheric [CO_2] from 417 ppm to 550 ppm (the former already elevated compared to pre-industrial values (278 ppm) (IPCC, 2007b; Le Quéré *et al.*, 2009)), and an increase in the frequency and severity of droughts and heat waves (IPCC, 2014). Meta-analysis of wheat productivity data has shown a trend of decreased grain yield with increasing temperature (Asseng *et al.*, 2015). It has been suggested that this loss of yield may be offset by the simultaneous rise in atmospheric [CO_2] (Parry *et al.*, 2004) and a previous study has shown that increasing atmospheric [CO_2] can buffer against heatwaves in semi-arid environments (Fitzgerald *et al.*, 2016). However, in contrast, recent field trials of the C3 model plant soybean demonstrated that elevated atmospheric [CO_2] is insufficient to rescue yield losses caused by drought conditions from increased temperature (Gray *et al.*, 2016), indicating that increases in [CO_2] cannot be assumed to provide the single solution to mitigate against the predicted global temperature increase.

In an effort to tackle this problem effectively, a large body of research has been presented showing that the genetic manipulation of carbon assimilation, by over-expressing proteins/enzymes of the Calvin-Benson-Bassham Cycle (CBC), photorespiration, the xanthophyll cycle, transport proteins, and/or Electron Transport (ET), can increase plant productivity and yield potential of both model and crop species (for comprehensive reviews see (Simkin *et al.*, 2019; Weber and Bar-Even, 2019; Simkin, 2019; South *et al.*, 2018). Other approaches for improving photosynthesis include, but

are not limited to, engineering C4 photosynthesis in C3 plants (Schuler *et al.*, 2016; Y., Li *et al.*, 2017), engineering bacterial carbon concentrating mechanisms and pyrenoids into C3 plants (Long *et al.*, 2018; Atkinson *et al.*, 2020) and the introduction of photorespiratory bypasses (Dalal et al., 2015; Kebeish et al., 2007; Nölke et al., 2014; South et al 2018). It is hoped that these approaches will create "future-proofed" plants, capable of feeding a growing populous while exploiting predicted environmental changes.

While this has been explored in a range of staple crops, research in this field on fruit crops has so far been lacking. This thesis will therefore investigate the potential of genetic manipulation of photosynthesis in the cultivated strawberry (*Fragaria x ananassa Duch.*). Fruiting crops have been typically understudied in this area of research, despite their high value and potential to combat micronutrient deficiency. This thesis aims to understand what the species-specific limiting factors are on strawberry photosynthesis via exploration of how photosynthesis and fruit yield segregates across polytunnel rows. It then describes work undertaken to transform strawberry with photosynthetic genes of interest in an effort to increase photosynthetic efficiency. Finally, changes in photosynthesis are determined via chlorophyll fluorescence in an effort to understand how these transformations affect the dynamics of the photosynthetic electron transport chain. Overall, this thesis aims to provide groundwork that can be applied to future studies into strawberry photosynthesis for enhancement of fruit yield and flavour traits.

This chapter evaluates the current literature surrounding genetic improvement of photosynthesis and questions how this may be applied to fruiting crops using growth in carbon-enriched atmospheres as a proxy for genetically improved photosynthesis. More specific research pertaining to cultivated strawberry is described where relevant in Chapters 3 – 5.

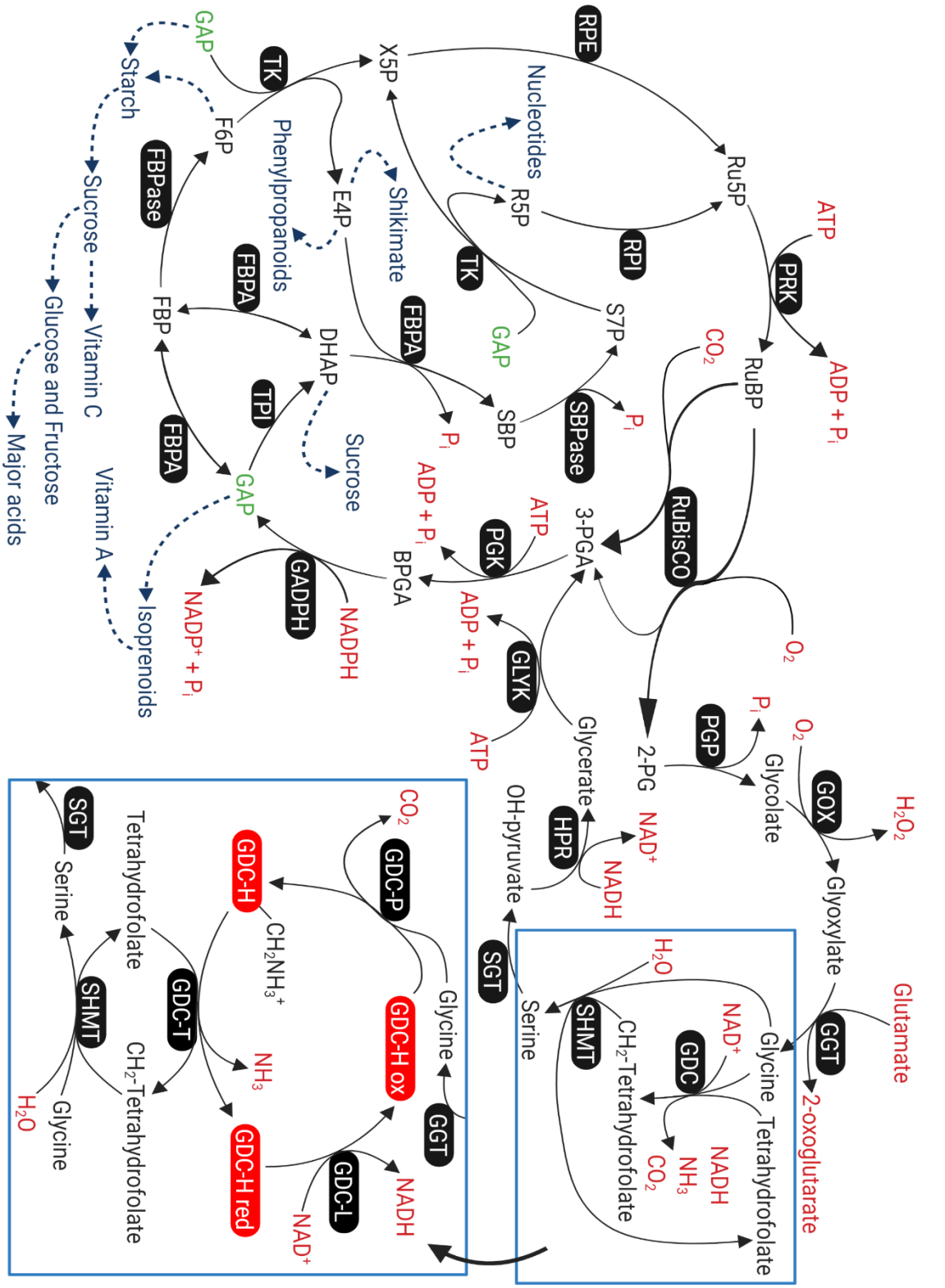

Fig. 1.1: Description provided on next page.

Fig. 1.1: Schematic Representation of Photosynthesis and Photorespiration. Sedoheptulose-1,7-bisphosphatase (SBPase: EC.3.1.3.37), fructose-1,6-bisphosphate aldolase (FBPA: EC.4.1.2.13), fructose-1,6-bisphosphatases (FBPase: EC.3.1.3.11), triosephosphate isomerase [TPI: EC 5.3.1.1], transketolase (TK: EC.2.2.1.1), ribulose-5-phosphate 3-epimerase (RPE: EC.5.1.3.1), ribulose-5-phosphate isomerase (RPI: EC.5.3.1.6) phosphoribulokinase (PRK: EC.2.7.1.19), phosphoglycerate kinase (PGK: EC.2.7.2.3), ribulose-bisphosphate carboxylase (RuBisCO: EC.4.1.1.39), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Glycolate oxidase (GOX; EC.1.1.3.1), 2-phosphoglycerate phosphatase (PGP; EC.3.1.3.13), serine-glyoxylate transaminase (SGT; EC 2.6.1.45), glycine:2-oxoglutarate aminotransferase (GGT; EC.2.6.1.4), glycerate-3-kinase (GLYK; EC.2.7.1.31), hydroxypyruvate reductase (HPR; EC.1.11.81), glycine decarboxylase (GDC), aminomethyltransferase (GDC-T: EC.2.1.2.10), glycine decarboxylase (GDC-P: EC.1.4.4.2), dihydrolipoyl dehydrogenase (GDC-L: EC.1.8.1.4), glycine decarboxylase H-protein (GDC-H), serine hydroxymethyltransferase (SHMT; EC 2.1.2.1). Created with BioRender.com.

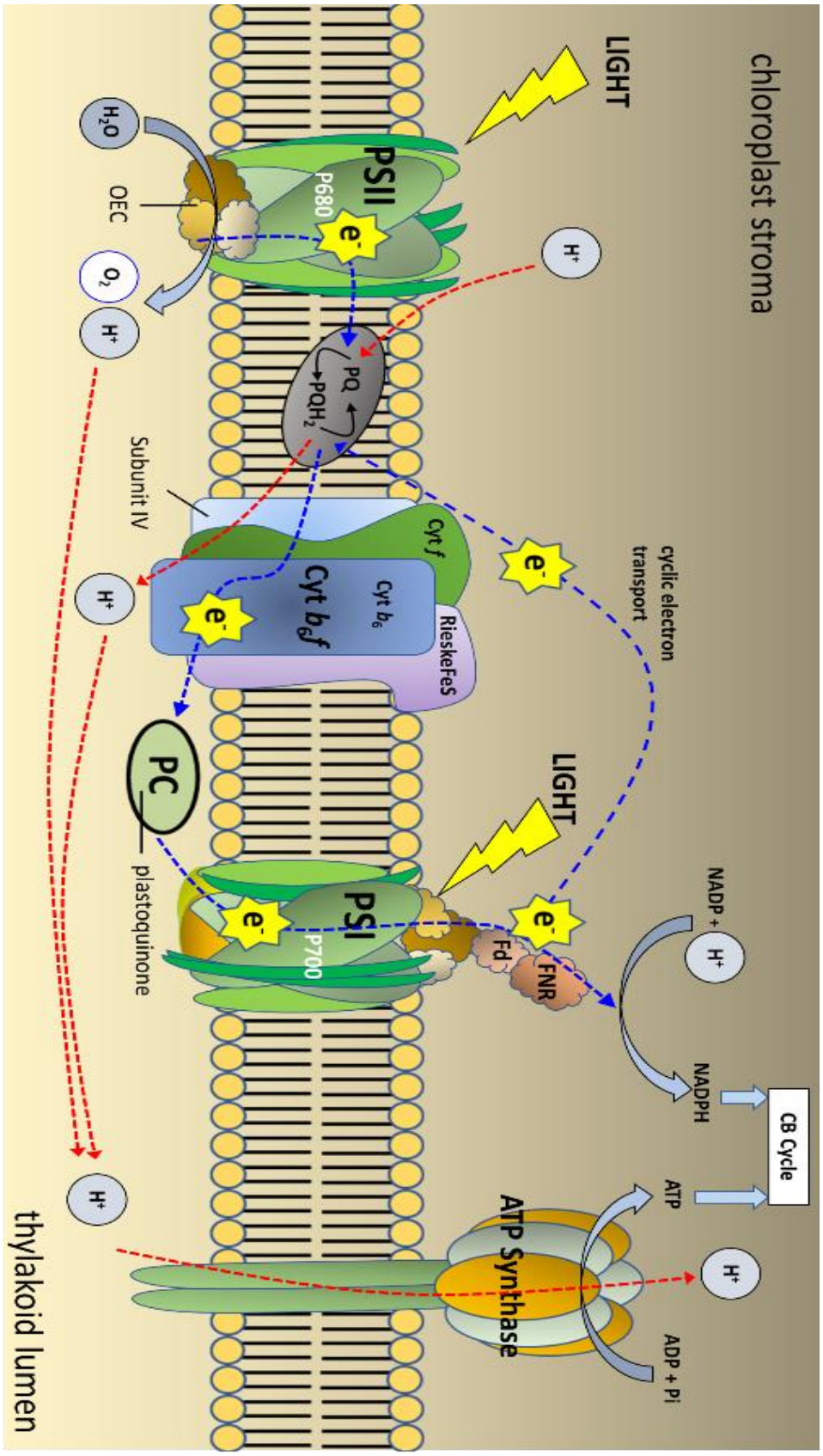

Fig. 1.2: Description provided on next page.

Fig. 1.2: Schematic Representation of Photosynthetic Electron Transport. Photosystem I (PSI), Photosystem II (PSII), Cytochrome b_6f complex (Cyt b_6f) (Cytochrome b_6, RieskeFes Cytochrome f), plastocyanin (PC), Ferredoxin (Fd) and ferredoxin-NADP reductase (FNR). The oxygen evolving complex (OEC), Plastoquinone (PQ) and ATP Synthase are shown. Cyanobacterial cytochrome c_6 (Cyt c_6), transfers electrons from the Cyt b_6f complex to PSI at a faster rate than observed for PC, plant ferrodoxin-like protein (PFLP). Adapted with permission from Simkin, 2019.

1.1.2 The components of photosynthesis

Photosynthesis is divided into two major processes – the harvesting of light energy via the photosynthetic electron transport chain (ETC) and the fixing of atmospheric CO_2 into biomass via the Calvin-Benson-Bassham Cycle (CBC). These processes are described in detail in Fig. 1.1 – 1.2. In brief, light is absorbed by light harvesting complexes of the ETC and the energy transferred to a chlorophyll reaction centre. This excites a "special pair" of electrons which enables the transfer of electrons along a series of electron carriers. These transfers facilitate the creation of a proton gradient to generate ATP for chemical energy and the generation of NADPH, a reducing co-factor. ATP and NADPH are then used to drive the CBC, where atmospheric CO_2 is fixed by RuBisCO to generate carbon intermediates that can be used to generate biomass. The rate of electron transfer and the rate of RuBisCO carboxylation can be measured and compared by recording how carbon assimilation varies with carbon concentration in the leaf mesophyll (i.e. an A-Ci curve). These rates are termed J_{max} and V_{cmax} respectively (Walker *et al.*, 2014). Throughout this thesis, the terms rate, capacity, and efficiency will be used. Photosynthetic rate is defined as how much photosynthesis can occur over a set time period; photosynthetic capacity is defined as the maximum amount of photosynthesis that can be carried out (be it in relation to the whole plant or on a leaf-by-leaf basis); and photosynthetic efficiency is defined as how much photosynthesis is being carried out relative to energetic losses and environmental conditions.

Many factors exist that limit and regulate photosynthetic physiology and these vary with environmental conditions although, broadly speaking, photosynthetic rate is greatest when not limited by availability of light, atmospheric [CO_2], water, and soil nutrients (particularly nitrogen). Under conditions of low atmospheric [CO_2] and/or high

irradiance, RuBisCO is rate-limiting due to less available substrate and a competing oxygenation reaction (see more details in 1.3) but under conditions of high atmospheric [CO_2] and/or low irradiance, regeneration of the carbon acceptor RuBP becomes rate-limiting due to depletion of the RuBP pool (exact numbers vary depending on degree of irradiance vs atmospheric [CO_2]) (von Caemmerer and Evans, 2010). Irradiance is also limiting on the ETC, since reduced availability of photosynthetically active radiation (PAR) reduces the quantity of light absorbed, thus reducing the quantity of electron transfer. This makes measurement of J_{max} and V_{cmax} valuable for unpicking where limitation is occurring. Furthermore, RuBisCO is only catalytically active in conjunction with RuBisCO activase (RCA), a highly heat-labile protein – this makes RuBisCO activity very sensitive to temperature and can limit photosynthesis under high temperature conditions (Singh *et al.*, 2014). Molecular physiology is not the only area to consider: leaf morphology and canopy architecture can constrain received light; biotic stresses can negatively feedback onto photosynthesis in a similar manner to the abiotic stresses previously discussed; controlled conditions can create more favourable, "pampered" environments for photosynthesis compared to "pestered" field conditions; and greater photosynthesis can provide more biomass for growth of more green tissue, enabling exponentially greater photosynthesis in plants that achieve high levels of photosynthesis earlier in development. Unpicking these many factors make improving photosynthesis highly complex and demonstrates the need for tailored solutions depending on crop variety, geographical location and local environment.

1.2. Improving photosynthesis through genetic modification the Calvin-Benson-Bassham Cycle

Several models predict yield gains are limited by photosynthetic efficiency, although this is complicated by limiting factors such as crop type, environment and water availability (Wu *et al.*, 2019). Furthermore, a meta-analysis of APSIM models (https://www.apsim.info/) in wheat did not define photosynthetic efficiency as a major limiting factor on yield (though water availability of atmospheric [CO_2] were defined as important) (Hao *et al.*, 2021). Despite this, a large body of evidence has developed demonstrating that improving the function of the photosynthetic process is a tractable approach for improving crop yields. This can be achieved through conventional breeding (Fischer *et al.*, 1998) but has not been consistently selected for (Theeuwen *et al.*, 2022). Due to the pressing global food crisis and speed of genetic technologies compared to conventional breeding, genetic engineering methods must be considered as an alternative to increase photosynthetic rates of crop plants. In an agricultural context, genetically modifying crops to improve photosynthesis has the potential to reduce time to harvest, which could increase seasonal and yearly output for growers, though this would need to be considered in the context of temperature and species-specific development. This approach could also make crops more resilient to the impacts of climate change, such as shorter growing seasons and extreme weather events occurring towards the end of current growing seasons, and make crops more suitable for growing at a higher latitude (see 1.7). The genetic knowledge gained from the studies outlined below can also be used to inform marker assisted and genomic selection breeding strategies for enhanced photosynthesis.

1.2.1. Modelling the Calvin-Benson-Bassham Cycle

Several methods exist for identifying targets for improving photosynthetic efficiency, such as: computational modelling of metabolism to predict bottlenecks in flux through desirable pathways; functional knockdown studies of predicted targets to confirm or deny predicted changes on photosynthetic efficiency; upregulation of photosynthesis associated proteins to evaluate changes in photosynthetic efficiency; and physiological analysis of models to understand how perturbations influence photosynthesis (e.g. A-Ci analysis to determine J_{max} and V_{cmax}). Both methods have been used to produce a suite of potential targets (**Table 1.1**). The CBC has been modelled extensively (for full review and comparison of these models, see (Arnold and Nikoloski, 2011; Jablonsky *et al.*, 2011). A large subset of these models focus on the carbon fixation process by RuBisCO and condense the remaining steps. This makes the model smaller, generating a more robust system to make predictions from. Indeed, Arnold and Nikoloski et al., (2011) rank the model by Farquhar et al., (1980), a RuBisCO focussed model, to be the best model of the CBC available in the current literature (Farquhar *et al.*, 1980). However due to the condensed nature of these models, they are less suitable for making predictions for metabolic engineering. Six models by Giersch et al. (1990), Hahn (1986), Laisk et al. (2006), Poolman et al. (2000) and Zhu et al. (2007, 2009) use greater detail for predicting effects of metabolic engineering. The earlier models by Hahn (1986) and Giersch et al. (1990) lack sufficient detail of important metabolic interactions to make accurate predictions for modern day metabolic engineering and Laisk et al. (2006) and Zhu et al. (2009) only consider naturally occurring steady state dynamics of the system, eliminating the possibility of understanding non-natural dynamics that could be achieved through metabolic engineering (although Zhu et al., (2009) does evaluate how the steady state may change with varying external conditions, which could act as a proxy for metabolic engineering). This leaves a handful

of models which are appropriate for understanding targets for metabolic engineering. Poolman et al. (2000) uses metabolic control analysis to identify bottlenecks in the CBC and identifies that flux is strongly regulated by SBPase. Zhu et al. (2007) uses an evolutionary algorithm over 1500 generations to redistribute proteinaceous nitrogen of CBC enzymes to generate a hypothetical photosynthetic system with maximised photosynthetic efficiency. This model is able to capture the range of enzyme concentration changes that need to occur for maximised photosynthesis – this includes increased concentrations of SBPase, FBPA and the non-CBC starch synthesis enzyme adenosine diphosphate glucose pyrophosphorylase (AGPase). This would suggest that these enzymes are strong potential targets for overexpression. Morales *et al.*, 2018 explored the effect of sunflecks in their dynamic model, making these results particularly relevant in the context of field grown crops. This model found that stomatal conductance and photoprotective mechanisms, such as the movement of chloroplasts and relaxation of NPQ, could be greatly limiting towards optimal photosynthetic efficiency. The model also found that reactions which catalyse the regeneration of RuBP in the CBC (such as the one catalysed by SBPase) were rate-limiting in extended sunflecks (> 4s). This suggests there are metabolic, cellular and mechanical bottlenecks limiting photosynthesis in C3 plants.

Protein/Enzyme Name	Role	Activity *in planta*
SBPase	Calvin-Benson Cycle	Catalyses formation of S7P from SBP
FBPA	Calvin-Benson Cycle	Catalyses formation of DHAP from G3P via FBP Catalyses formation of SBP from E4P and DHAP
FBPase	Calvin-Benson Cycle	Catalyses formation of F6P from FBP
cySBPase	Calvin-Benson Cycle	Cyanobacterial enzyme catalyses formation of S7P from SBP
cyFBPase	Calvin-Benson Cycle	Cyanobacterial enzyme catalyses formation of F6P from FBP
FS Bif	Calvin-Benson Cycle	Cyanobacterial enzyme Catalyses same reactions as SBPase and FBPase
TK	Calvin-Benson Cycle	Catalyses formation of X5P and R5P from G3P and F6P
ictB	Carbon transport?	First identified from a cyanobacterium (Synechococcus sp. PCC 7942). Activity currently unknown
GCS Proteins	Photorespiration	H, L and P-protein catalyse the formation of serine from glycine
Cytochrome c_6	Electron transporter	In cyanobacteria and green algae, Cytochrome c6 (cyt c6) has been shown to replace PC as an electron carrier in in response to copper deficiency
RieskeFeS protein	Component of the Cytochrome b6f complex of electron transport	Perform redox reactions to transfer electrons along chloroplastic ETC Act as signal to drive formation of chloroplastic ETC complexes

Table 1.1: Single gene targets to be manipulated for improved photosynthesis. Sedoheptulose-1,7-bisphosphatase (SBPase); fructose-1,6-bisphosphate aldolase (FBPA); Bifunctional FBP/SBPase (FS Bif); Glycine decarboxylase H protein Cytochrome c6 (Cyt c6); inorganic carbon transporter B (ictB); and the cyanobacterial enzymes SBPase (cySBPase), FBPase (cyFBPase) and the bifunctional FBP/SBPase (FS Bif)

1.2.2. Genetic manipulation of Calvin-Benson-Bassham Cycle increases carbon assimilation and yield

Knockdown experiments in tobacco (*Nicotiana tabacum*) to reduce SBPase gene expression demonstrated that small reductions in SBPase activity (> 9%) result in a reduction in carbon assimilation (A_{max} reduced by > 12 %), growth rates and biomass yields (Harrison *et al.*, 1998; Harrison *et al.*, 2001; Lawson *et al.*, 2006; Olcer, 2002). These data demonstrate that SBPase exerts strong regulation over CBC flux, identifying it as a good target for overexpression. It was found that an SBPase activity increase of > 1.3-fold was necessary to see biomass increases in transgenic tobacco in controlled, ambient [CO_2] conditions with 400 µmol m^{-2} s^{-1} light intensity compared to an FBPase activity increase of > 1.7-fold (Tamoi *et al.*, 2006). This helps to underline the small increase in enzyme activity needed for SBPase overexpression to produce yield gains in a controlled environment (Driever *et al.*, 2017; Lefebvre *et al.*, 2005). Knockdown of FBPA in potato found that lines with a greater than 50 % reduction in FBPA activity exhibited reduced biomass phenotypes (Haake *et al.*, 1998; Haake *et al.*, 1999), suggesting FBPA is also a suitable target for photosynthetic manipulation.

Several studies have overexpressed the target enzyme SBPase, in an effort to increase plant biomass (**Fig. 1.1; Supplemental Table 6**). In the model plant *Arabidopsis thaliana* (Arabidopsis), a 42 % increase in plant biomass for transgenic plants compared to WT was reported at the growth stage when inflorescence is first visible and a 14 % – 23 % increase in biomass (dependent on growth conditions) was reported at seed harvest. A 39 % – 53 % increase in seed yield was also observed (Simkin *et al.*, 2017a). In tobacco, constitutive overexpression of SBPase resulted in a 40 % increase in shoot biomass under controlled greenhouse conditions (Lefebvre *et al.*, 2005). These same plants were grown by Simkin et al., (2015) and the same biomass increase reported,

demonstrating that this yield increase was conserved over 4 to 6 generations. However, over-expression of SBPase in *N. tabacum* grown in field conditions did not result in an increase in biomass under ambient [CO_2], although a significant increase in biomass was reported for field grown transgenic plants exposed to saturating [CO_2] (585 ppm [CO_2]) (Rosenthal *et al.*, 2011), indicating the potential of this work for improving crop productivity as atmospheric [CO_2] levels increase due to climate change; an increase up to at least 585 ppm atmospheric [CO_2] is predicted to occur before the end of the century (according to IPCC models) (IPCC Data Distribution Centre, 2001). With a current annual increase in atmospheric [CO_2] of 2.5 ppm per annum, atmospheric [CO_2] could reach 585 ppm by 2088. SBPase has since been overexpressed in the cereal crop wheat – biomass yield increases of 14 % – 31 % were reported and an up to 40 % increase in grain yield in glasshouse conditions (Driever *et al.*, 2017). Work carried out in tobacco reported that plants expressing cyanobacterial SBPase (cySBPase) showed a 20 % increase in the rate of photosynthetic CO_2 fixation and a 50 % increase in final biomass compared to controls. Constitutive overexpression of FBPA in tobacco led to a 10 % – 30 % increase in aerial plant biomass under ambient [CO_2] and a 70 % – 120 % increase under elevated [CO_2] (700 ppm); the latter condition was also reported to improve carbon assimilation by 50 % (Uematsu *et al.*, 2012). Overexpression of FBPA in Arabidopsis resulted in a 25 % – 29 % increase in dry weight and a 35 % – 36 % increase in seed yield (Simkin *et al.*, 2017a). FBPA overexpression therefore improves photosynthesis and yield in a similar manner to SBPase over-expression (**Fig. 1.1; Supplemental Table 6**).

To date, only one study in a fruiting crop (tomato) evaluating the manipulation of photosynthesis by overexpressing of SBPase (see **Supplemental Table 6**) has been published (Ding *et al.*, 2016), demonstrating that SBPase overexpression can improve photosynthesis and biomass across species and importantly in fruiting crops. These

authors showed that increasing SBPase activity in tomato increased photosynthesis by 20 % – 25 %, increased vegetative biomass by 30 %, resulted in early onset flowering and conferred tolerance to chilling stress (Ding *et al.*, 2016). However, these authors did not report if increasing SBPase and photosynthetic efficiency increased fruit yield. Given that fruit are a carbon priority, at least in crop trees (Génard *et al.*, 2008), one would expect that increasing carbon assimilation would increase carbon availability for fruit set and fruit quality, as carbon determines the size of the tissues and their nutritional composition (i.e. sugars, acids, and other biochemical compounds, such as carotenoids, that contribute to the flavour and aroma of fruits (Auldridge *et al.*, 2006; Simkin *et al.*, 2004)).

1.3. CO_2 enrichment – a proxy for genetically enhanced photosynthesis in fruiting crops

Genetic manipulation of photosynthesis fruiting crops has only been explored, so far, in tomato (Ding *et al.*, 2016) and cucumber (Bi *et al.*, 2013) and yield increases were achieved in both of these plant species. However, neither paper reported on fruit quality. Therefore, to understand better how genetic manipulation of photosynthesis may affect fruit yield and fruit quality in a wide range of crops, a proxy is needed. Elevated atmospheric [CO_2] has been shown to increase photosynthesis and biomass in a wide range of plant species, including several fruiting crops (Ainsworth and Long, 2005; Ainsworth and Long, 2020), and could therefore make a proxy for understanding genetic enhancement of photosynthesis.

CO_2 enters photosynthesis at the Calvin-Benson-Bassham Cycle – RuBisCO fixes atmospheric carbon using the 5-carbon compound ribulose-1,5-bisphosphate (RuBP) as a carbon acceptor (see **Fig. 1.1**). However, in approximately 25 % of reactions (Peterhansel *et al.*, 2010), O_2 is fixed instead, resulting in the production of the toxic compound 2-phosphoglycolate (2-PG). Subsequent processing of this compound, a metabolic pathway known as photorespiration, results in the production of 1 mole of 3-phosphoglycerate (3-PGA) and 1 mole of CO_2 lost for every 2 moles of 2-PG processed (Bauwe *et al.*, 2010). This makes RuBisCO far less efficient. However, when exposed to an atmosphere with increased [CO_2] RuBisCO rates for the desirable CO_2 fixation reaction are increased due to a greater availability of the CO_2 substrate (Sage *et al.*, 2008). As RuBisCO is often rate-limiting on the CBC (Parry *et al.*, 2013), elevation of atmospheric [CO_2] can increase photosynthetic rate in a similar manner to improving photosynthesis through genetic manipulation of the CBC. It should be noted, however, that this assumes that improvement of CBC efficiency (and associated carbon

assimilation) has similar effects on plant growth and development regardless of which element of the CBC has been targeted. This is a significant assumption to make, since it has been demonstrated that different species react differently to the same genetic manipulation (see 1.8) and does not account for differences in metabolic regulation that may occur as a result of different manipulations. Despite this, elevated atmospheric [CO_2] does still result in a demonstrated increase in carbon assimilation and has been well studied in many fruit crops, making it a reasonable proxy for predicting how genetic manipulation of photosynthesis may affect fruit crop traits. This section explores how the yield and quality of fruiting crops changes when grown in a carbon-enriched atmosphere and uses this to hypothesise the effects of genetic manipulation to increase photosynthetic rate and CO_2 assimilation of these same crops.

1.3.1. Free-Air Carbon Enrichment (FACE) studies

The majority of research evaluating *e*[CO_2] for fruit crop production has been carried out in controlled environment conditions (chambers) and commercial greenhouses where crops are grown in elevated [CO_2], however some work has been done on field-grown fruit crops with free-air carbon enrichment (FACE).

FACE is a technique used to explore how field-grown plants respond to growth in atmospheric *e*[CO_2] without altering the plants' local climate (see **Supplemental Table 7**). Field-grown plants remain exposed to a dynamic environment with stresses that are removed in controlled environment conditions, such as fluctuations in weather and biotic stress. This can give a better prediction of how typically field-grown crops may perform in true agricultural practice; indeed, a study by (Poorter *et al.*, 2016) found that the correlation coefficient between plants grown in controlled carbon-enriched atmospheres and plants grown in FACE conditions for a selection of key phenotypes was only 0.26 (Poorter *et al.*, 2016). As many fruit crops are grown in the field, it is important to consider how external factors may influence harvest yield and quality for plants with increased photosynthesis through FACE so that the effects of growth in dynamic conditions can be extrapolated to transgenic plants with genetically manipulated photosynthesis. For a recent review of FACE studies, see Ainsworth and Long, (2020).

FACE studies have largely focused on cereal and staple crops, including wheat, rice, maize, barley, sorghum, soybean, potato, cassava, lentil and chickpea (Ainsworth and Long, 2005; Miglietta *et al.*, 1998; Weigel and Manderscheid, 2012; Leakey *et al.*, 2006; Morgan *et al.*, 2005; Rosenthal *et al.*, 2012; Singh *et al.*, 2013; Bourgault *et al.*, 2018), however some research has been carried out on horticultural crops, including oilseed rape, sugar beet, field pea, faba bean, coffee, mung bean, mustard, peanut and grape (Weigel and Manderscheid, 2012; Singh *et al.*, 2013; Högy *et al.*, 2010; Bourgault *et al.*, 2016; Parvin *et al.*, 2019; Ghini *et al.*, 2015; Bindi *et al.*, 2001). Of these, grape is

the only fruit crop to have been studied. Work has been carried out on both red (Sangiovese, Cabernet Sauvignon) and white (Riesling) cultivars under FACE conditions ($e[CO_2]$ = 550 ppm and 700 ppm), and demonstrated increases in carbon assimilation by up to 39.7 % in Riesling and up to 43.3 % in Cabernet Sauvignon. This resulted in a total fruit yield increase, through increased bunch weight, of up to 17.8 % in Riesling and up to 17.3 % in Cabernet Sauvignon (see **Supplemental Table 7**). Additionally, there was an increase in total sugar yield of up to 14.9 % in Riesling and 13.6 % in Cabernet Sauvignon, however there was a slight reduction in the sugar content of must (< 3.6 %) (Wohlfahrt *et al.*, 2018). No significant difference was seen between treatments, suggesting that atmospheric $[CO_2]$ is saturating for grape at 550 ppm. In the cultivar Sangiovese grown under FACE conditions (atmospheric $e[CO_2]$ = 550 and 700 ppm), a 35 % – 45 % increase in berry yield was reported, however mature berries did not contain significantly different quantities of sugars and acids compared with grapes grown under ambient atmospheric $[CO_2]$ (Bindi *et al.*, 2001). An increase in the total flavonoid content (35 %) and anthocyanin content (23 %) of the wine produced was reported however, leading to a 41 % increase in colour intensity (Bindi *et al.*, 2005). Growth in a free air carbon-enriched atmosphere can therefore affect a wide range of grape quality traits and appears to be cultivar dependent.

Taken together, these studies demonstrate that $e[CO_2]$ can improve photosynthetic, yield and quality traits of a fruit crop simultaneously in a dynamic environment in a cultivar dependent manner. Extrapolating this to genetic manipulation of photosynthesis, it is possible that, for field grown crops, this technique could generate similar improvements in these key traits to that observed in FACE studies (with the added benefit of not requiring supplemental CO_2 regimens). This is an exciting prospect, as yield and quality traits have often been difficult to breed for

simultaneously. Hence, genetic manipulation of photosynthesis could be a long-term solution for solving this breeding conundrum.

1.3.2. Cultivating fruiting crops in elevated [CO_2] increases carbon assimilation rates, yield and quality

1.3.2.1 Tomato and other solanaceous crops

Improved photosynthesis in a carbon enriched atmosphere has been demonstrated in tomato, sweet pepper and chili pepper (Hicklenton and Jolliffe, 1978; X., J., Li *et al.*, 2017; Nilsen *et al.*, 1983; Pazzagli *et al.*, 2016; Manuel E. Porras *et al.*, 2017; Yelle *et al.*, 2019) and has been linked to improved yield. Commercially, tomato (*Solanum lycopersicum*) crops are grown in greenhouses with *e*[CO_2], in some cases as high as 2000 ppm and has been extensively studied (Fierro *et al.*, 1994; Hicklenton and Jolliffe, 1978; Islam *et al.*, 1996; Khan *et al.*, 2013; Kimball and Mitchell, 1978; Li *et al.*, 2007; Li *et al.*, 1999; Mamatha *et al.*, 2014; Nilsen *et al.*, 1983; Ozcelik and Akilh, 1999; Pazzagli *et al.*, 2016; Peet and Willits, 1984; Reinert *et al.*, 1997; Sánchez-González *et al.*, 2016; Tripp *et al.*, 1991; Wei *et al.*, 2018; Yelle *et al.*, 2019; J., Liu *et al.*, 2020; Nederhoff, 1994; Hartz *et al.*, 2019; Zhang *et al.*, 2014; Wheeler *et al.*, 1997; Helyes *et al.*, 2011). Under *e*[CO_2], tomato fruit yield increases ranged from 7 % – 125 % for plants grown in carbon-enriched greenhouses (see **Supplemental Table 1**; [CO_2] ranged from 450 ppm – 1200 ppm) compared with plants grown under ambient [CO_2] (*a*[CO_2]). An increase in the quantity of non-reducing sugars (glucose and fructose) was broadly reported (Islam *et al.*, 1996; Khan *et al.*, 2013; Li *et al.*, 2007; Sánchez-González *et al.*, 2016; Zhang *et al.*, 2014) and fully ripe tomatoes grown in an *e*[CO_2] were found to be preferable for consumption in sensory panels (Zhang *et al.*, 2014). As liking sweetness has been shown to be a universal trait (Drewnowski *et al.*, 2012), it is possible that this increase in sugar is responsible for preference of the carbon enriched tomato fruits. An increase in vitamin C was also found between most studies (Islam *et al.*, 1996; Li *et al.*, 2007; Mamatha *et al.*, 2014; Zhang *et al.*, 2014; Karim *et al.*, 2020), potentially

improving the health benefit gained from consumption of carbon-enriched grown tomatoes (**Supplemental Table 1**). Similarly, tomato fruit concentration of lycopene and β-carotene (pro-vitamin A) were found to have increased by as much as 30 % and 70 % respectively in some studies (Zhang *et al.*, 2014). Rangaswamy et al. (2021), for example, found an increase in carotenoid (+20 %) and lycopene (+31 %) in the tomato fruit of plants grown at *e*[CO_2] of 550 ppm, however a decrease in carotenoid content (- 12%) was observed when grown at *e*[CO_2] of 700 ppm. Carotenoids are also the precursors of several flavour and aroma compounds. β-carotene, the precursors for the formation of the aromatic apocarotenoid β-ionone is cleaved by carotenoid cleavage dioxygenases CCD1 and CCD4 (Simkin *et al.*, 2004; Zhang *et al.*, 2016). Furthermore, lycopene is cleaved by CCD1 to form several important flavour and aroma compounds including 6,10-dimethyl-3,5,9-undecatrien-2-one (pseudoionone; (Simkin *et al.*, 2004)), 6-methyl-5-hepten-2-one (MHO; (Vogel *et al.*, 2008)) and geranial (Ilg *et al.*, 2009). MHO has been shown to be an important contributor to tomato fruit flavour (Baldwin *et al.*, 2000; Buttery *et al.*, 1990) and has also been shown to accumulate in tomato fruit with higher lycopene levels (Simkin *et al.*, 2007). This phenomenon deserves further study to better understand the relationship between carotenoid content and CO_2 assimilation potential. These data suggest that genetic manipulation of photosynthesis is a viable method for increasing CO_2 assimilation under *a*[CO_2] in tomato, improving fruit yield and fruit quality with the added of potential of removing a growers requirements for growing crops in *e*[CO_2] and/or giving crops a higher potential of maximising CO_2 use *e*[CO_2]). It should also be noted that tomato fruit are green and photosynthetically active themselves, thereby contributing to fruit size and quality (for review see (Simkin *et al.*, 2020)).

Other studies have been carried out in sweet pepper (Nederhoff, 1994; Alonso *et al.*, 2012; Aloni and Karni, 2002; Fierro *et al.*, 1994; Milhet and Costes, 1975; Penuelas

et al., 1995; Piñero *et al.*, 2018; M. E. Porras, Lorenzo, *et al.*, 2017; M. E. Porras, Medrano, *et al.*, 2017; Rezende *et al.*, 2003), chili pepper (X., J., Li *et al.*, 2017; Garruña-Hernández *et al.*, 2013; Andaleeb *et al.*, 2017; Das, Das, Kalita and Baruah, 2020; Das, Das, Kalita and Bharali, 2020; Das *et al.*, 2016; Garruña-Hernández *et al.*, 2012) and eggplant (Nederhoff, 1994; Milhet and Costes, 1975; Imazu *et al.*, 1967).

In sweet pepper (*Capsicum annum*), for *e*[CO_2] in the range of 450 ppm – 1000 ppm, a fruit yield increase of 12.9 % – 370.2 % was reported (**Supplemental Table 2**). This large range of yield increases derives from a single study of sweet pepper grown under *e*[CO_2] of 1000 ppm (Milhet and Costes, 1975), with most other studies reporting yield increases in the range of 12.9 % – 47.4 % in the absence of other parameters (Nederhoff, 1994; Alonso *et al.*, 2012; Aloni and Karni, 2002; Piñero *et al.*, 2018; M. E. Porras, Lorenzo, *et al.*, 2017; M. E. Porras, Medrano, *et al.*, 2017; Rezende *et al.*, 2003). Yield was also found to vary with different irrigation programmes (Penuelas *et al.*, 1995; Rezende *et al.*, 2003), nitrogen sources (Penuelas *et al.*, 1995), substrate salinity (Piñero *et al.*, 2018; M. E. Porras, Lorenzo, *et al.*, 2017) and pruning regimens (Alonso *et al.*, 2012). Interestingly, optimal [CO_2] for maximum yield varied between different irrigation programmes (Rezende *et al.*, 2003). Growth in at ~800ppm *e*[CO_2] was found to reduce sweet pepper total amino acid content by up to 29 %, including reductions in the sweet tasting amino acids alanine and glycine, which could be detrimental to the perceived fruit flavour (Piñero *et al.*, 2018). Given that previous work in tomato has shown an increase in potential phytonutrients in fruit grown at 550ppm and a decrease in those grown at 700ppm, further research is needed to better identify the impact of carbon-enrichment on sweet pepper fruit quality.

In chili pepper (*Capsicum annum* and *Capsicum chinese*), yield increases of 43.8 % – 142 % were reported for *e*[CO_2] (in the range of 500 ppm – 1140 ppm). These yield increases were in part attributed to an increase in the size of fruits (Garruña-Hernández

et al., 2013). A 4°C increase in temperature was found to decrease yield even at *e*[CO_2] (750 ppm) (Das, Das, Kalita and Baruah, 2020; Das, Das, Kalita and Bharali, 2020), indicating that carbon enrichment is not sufficient to rescue yield in an extreme climate change scenario. Carbon-enriched growth was found to increase the capsaicinoid content of fruits, resulting in an increase in Scoville Heat Units (SHU) (Garruña-Hernández *et al.*, 2013; Das *et al.*, 2016). This approach therefore has potential for producing hotter varieties of chili, a growing and competitive market. However, vitamin C concentration of chili peppers was found to decrease by up to 15.84 % (Andaleeb *et al.*, 2017), reducing potential health benefits gained upon fruit consumption. This being said, it is difficult to draw real conclusions given the wildly different *e*[CO_2] levels tested in different studies (**Supplemental Table 2**) and the demonstration that even in the same plants, changing atmospheric [CO_2] concentrations can cause results to vary significantly.

In eggplant (*Solanum melongena*), yield increases of 23.6 % – 209.2 % were reported for *e*[CO_2] for the range 663 ppm – 3000 ppm. Studies differed in reporting the rate of eggplant development, with one paper reporting increased developmental rate during summer (Milhet and Costes, 1975) and another stating there was no change to developmental rate (Nederhoff, 1994). Better understanding of the rate of development is important for predicting how many harvests can be carried out over a year to increase annual yield and also for the development of varieties which extend the harvesting season so more crops can be planted. No studies have yet investigated any eggplant quality traits.

1.3.2.2. Strawberry and other rosaceous crops

Rosaceous crop research in this area has focused primarily on cultivated strawberry (Hartz *et al.*, 2019; Enoch *et al.*, 1976; Sun *et al.*, 2012; Himali N *et al.*, 2017; Chen *et al.*, 1997; Bunce, 2001; Bushway and Pritts, 2002; Keutgen *et al.*, 1997; Sung and Chen, 1991; Deng and Woodward, 1998; Lieten, 1997; Wang *et al.*, 2003; Wang and Bunce, 2004; Balasooriya *et al.*, 2019; Li *et al.*, 2020; Balasooriya *et al.*, 2018) with a small number of studies on raspberry (Percival *et al.*, 1996; Mochizuki *et al.*, 2010) and Nashi pear (Han *et al.*, 2012). This is likely due to the relatively smaller size and rapid growth of strawberry compared to other commercially important rosaceous fruit species, such as tree fruits, like apple and cherry, and woody stemmed shrub fruits, like raspberry and blackberry. This makes strawberry a convenient plant to study as a rosaceous model.

In cultivated strawberry (*Fragaria x ananassa Duch.*), reported yield increases ranged from 1.0 % – 51.0 % in plants grown under atmospheric *e*[CO_2] of 450 ppm – 3000 ppm (see **Supplemental Table 3**). This has been directly linked to an increased photosynthetic rate and an increased assimilation rate of CO_2 in strawberry leaves (Bunce, 2001; Bushway and Pritts, 2002; Keutgen *et al.*, 1997; Li *et al.*, 2020; Sung and Chen, 1991; Balasooriya *et al.*, 2018), with an assimilation rate increase of up to 73 % at optimal *e*[CO_2] of 600 ppm reported in one study (Keutgen *et al.*, 1997). This has been further investigated at a genetic level through RNA seq analysis; 150 genes were upregulated when strawberry plants were grown in an enriched-carbon atmosphere, with 14 of these being photosynthetic genes (Li *et al.*, 2020). Further annual yield increases could be achieved by an up to two week reduction in time to fruiting for plants grown in an enriched-carbon atmosphere (Enoch *et al.*, 1976; Bushway and Pritts, 2002). Several fruit quality traits were also improved by growth in greater atmospheric [CO_2]. Increases in reducing sugars, and therefore sweetness index, were reported (Sun

et al., 2012; Wang and Bunce, 2004) alongside reductions in organic acids (Wang and Bunce, 2004). This increase in sugar-acid ratio is highly favourable for a more pleasant perception of strawberry flavour (Drewnowski *et al.*, 2012). An increase in key volatile organic compounds, including furaneol, linalool and major esters, was also reported, further enhancing the "strawberry" flavour (Wang and Bunce, 2004). Growth in a carbon-enriched atmosphere therefore strongly enhances strawberry flavour. Additionally, vitamin C concentration was increased by up to 13.3 % alongside other antioxidant compounds (Wang *et al.*, 2003; Balasooriya *et al.*, 2019). Growth in carbon-enriched atmospheres therefore simultaneously improves yield, flavour and health benefits of strawberry fruits, creating enormous potential for strategies involving enhanced photosynthesis of strawberry plants, including genetic manipulation. This could be attributed to increased carbon assimilation leading to greater availability of CBC products for use in biosynthesis of biomass and secondary carbon metabolism (see Fig. 1.1 for an illustration of where these secondary products derive their carbon from the CBC).

In the rosaceous species raspberry (*Rubus idaeus*) and Nashi pear (*Pyrus pyrifolia*), there has been limited research. Tunnel grown raspberries were found to have yield enhanced by 12 % in a carbon-enriched atmosphere of, on average, 436 ppm CO_2 (Mochizuki *et al.*, 2010). Modelling of the carbon exchange rate of raspberry at a range of atmospheric [CO_2] found that [CO_2] explained 28 % of model variance, indicating that CO_2 exposure during growth has a large effect on this key photosynthetic parameter (Percival *et al.*, 1996). In Nashi pear, a carbon-enriched atmosphere of 700 ppm CO_2 was found to increase fruit weight, diameter and length with a 22.5 % increase in Brix, a key measure of sweetness for marketable fruit (Han *et al.*, 2012). While these studies are limited in what they report, they do indicate the high potential of carbon-enriched growth for improved photosynthesis, yield and quality of other rosaceous

species. Transformation methods have been reported in both raspberry and strawberry (Mathews, Wagoner, Cohen, *et al.*, 1995; Schaart, 2014; Wilson *et al.*, 2019), demonstrating the viability of a genetic approach for increased carbon uptake in rosaceous crops.

1.3.2.3. Cucumber and other cucurbitaceous crops

Cucumber is the most predominantly studied fruit crop of the cucurbitaceae in relation to growth in carbon-enriched atmospheres (Dong *et al.*, 2017; J., Dong *et al.*, 2018; J., long Dong *et al.*, 2018; Enoch *et al.*, 1976; Nederhoff, 1994; Heij and van Uffelen, 1984; Kläring *et al.*, 2007; Luomala *et al.*, 2008; Peet, 1986; Peet *et al.*, 1986; Sánchez-Guerrero *et al.*, 2009; Segura *et al.*, 2001; Hartz *et al.*, 2019; Sánchez-Guerrero *et al.*, 2005; FAO, 2017a) (see **Supplemental Table 4**). Improved carbon assimilation rates of up to 99 % and 112 % have been reported for cucumber and melon respectively when grown in a carbon-enriched atmosphere (Dong *et al.*, 2017; Mavrogianopoulos *et al.*, 1999), demonstrating that growth in $e[CO_2]$ improves photosynthesis of cucurbitaceous crops. In cucumber (*Cucumis sativus*), fruit yield increases for plants grown in enriched-carbon atmospheres ($[CO_2]$ = 450 ppm – 3000 ppm) ranged between 16.2 % and 41 % in the absence of other parameters that could alter fruit yield. In high nitrogen, fruit yield was as high as 106 % when grown under $e[CO_2]$ of 800 ppm (J., Dong *et al.*, 2018). Interestingly, for $e[CO_2]$ of 1200 ppm and high nitrogen treatment, studies found a yield increase between 71 % – 73 % (J., Dong *et al.*, 2018; Dong *et al.*, 2017), suggesting that an optimal concentration of atmospheric CO_2 exists for maximum yield returns. Unlike strawberry, no change in developmental rate was found for plants grown in a carbon-enriched atmosphere (Enoch *et al.*, 1976). There is large variation between studies on how cucumber fruit quality is impacted by carbon-enriched growth. Fructose and glucose were reported to increase by 6 % and 12 % in one study (J., Dong *et al.*, 2018) and by 75 % and 73 % respectively in another (J., long Dong *et al.*, 2018). The inorganic nutrient content of fruits was also reported to reduce for fruits grown in $e[CO_2]$, however only phosphorus showed a significant reduction in multiple cycles (Segura *et al.*, 2001). Understanding how growth in an enriched-carbon atmosphere in cucumber needs further study before strong conclusions can be drawn on how fruit

quality is affected, however these data do indicate that carbon-enriched growth may enhance fruit flavour and fruit yield at a cost of nutritional value. It should be noted that cucumber fruit remain green through to maturity, have stomata (suggesting they perform gas exchange to drive photosynthesis), and have a similar surface area to an expanded leaf (Sui *et al.*, 2017; Simkin *et al.*, 2020). It has previously been reported that cucumber fruit had high photosynthetic and respiratory rates (Todd *et al.*, 1961) and contribute approximately 9.4 % of their own carbon requirements (Sui *et al.*, 2017). In *e*[CO_2], it seems plausible that cucumber fruit photosynthesis may contribute directly to fruit size (and therefore yield by weight) and quality through their ability to directly access carbon in an enriched atmosphere.

In melon (*Cucumis melo*), yield increases in the range 7.3 % – 29.0 % were reported for atmospheric *e*[CO_2] of 800 ppm – 1200 ppm (Milhet and Costes, 1975; Mavrogianopoulos *et al.*, 1999). These increases were reduced by increased substrate salinity but fruit yield for these plants was still higher than for plants grown in high salinity at ambient atmospheric [CO_2], showing that increased atmospheric [CO_2] can somewhat mitigate yield losses from increased salinity (Mavrogianopoulos *et al.*, 1999). In tunnel grown summer squash (*Cucurbita pepo*), atmospheric *e*[CO_2] of 700 ppm – 1000 ppm was sufficient to increase marketable fruit yields by 16 % - 20 % (Hartz *et al.*, 2019). No studies have yet reported on the impact of carbon-enriched atmospheres on the quality or national value of melon or summer squash (see **Supplemental Table 4**).

Growing cucurbitaceous crops in carbon-enriched atmospheres clearly results in increased fruit yields for a range of crops, even if developmental rate is unchanged. Future work should now examine how fruit quality is changed by *e*[CO_2].Transformation methods exist for several members of the cucurbitaceae, including all crops mentioned in this review (Trulson *et al.*, 1986; Dong *et al.*, 1991; Shah *et al.*, 2008). This means that genetic manipulation of cucurbitaceous photosynthesis is possible and is hypothesised

to improve fruit yield across a range of crops, although it remains difficult to predict how this could impact on fruit quality.

1.3.2.4. Other fruit families

Effects of fruit growth in *e*[CO_2]have also been studied in grape (*Vitis vinifera*) (Kizildeniz *et al.*, 2015; Kizildeniz, Irigoyen, *et al.*, 2018; Kizildeniz, Pascual, *et al.*, 2018; Parra *et al.*, 2010) and orange (*Citrus* × *sinensis* and *Citrus x aurantium*) (Vu *et al.*, 2002; Kimball *et al.*, 2007; Idso *et al.*, 2002). Controlled environment studies in grape have focused on red and white varieties of the cultivar Tempranillo (see **Supplemental Table 5**). When grown in *e*[CO_2] of 700 ppm, no significant yield change was reported, though it is interesting to note that the red variety showed a small increase in bunch yield and the white variety a small decrease (Kizildeniz, Irigoyen, *et al.*, 2018). There has been extensive research examining how the quality of grape berries is additionally affected by temperature and irrigation in addition to elevated atmospheric [CO_2]. Across all climate regimens there was a small 2.6 % increase in Brix, however malic acid concentration was significantly decreased in poor irrigation and high temperature whereas tartaric acid concentration was significantly increased across all climate conditions (Kizildeniz, Pascual, *et al.*, 2018; Parra *et al.*, 2010). This suggests that synthesis of different organic acids in grape differs under *e*[CO_2] growth, similarly to reducing and non-reducing sugars in other crop species. Fruit anthocyanin content has been well studied, however most studies disagree on the effect *e*[CO_2] has (Kizildeniz *et al.*, 2015; Kizildeniz, Pascual, *et al.*, 2018). Interestingly, these studies differ considerably from FACE studies in grape, which provided considerably greater returns in both yield and quality parameters (see above).

Growth of sweet orange (*Citrus* × *sinensis*) in 720 ppm *e*[CO_2] increased the carbon exchange rate of leaves by up to 45 % and the water use efficiency (WUE) by up to 105 %, creating a clear link between growth in a carbon-enriched atmosphere and improved photosynthesis of orange (Vu *et al.*, 2002). A 17-yearlong study of sour orange (*Citrus x aurantium*) grown at an atmospheric [CO_2] of 300 ppm above ambient

conditions found an 85 % increase in fruit yield across the entire study, derived from an increase in fruit number rather than fruit size (Kimball *et al.*, 2007). Additionally, growth in the same atmospheric conditions was found to increase sour orange juice vitamin C concentration by 7 % (Idso *et al.*, 2002), potentially derived from increased available products from the CBC for secondary carbon metabolism. Together, this shows that growth in a carbon enriched atmosphere could provide large returns for both yield and nutritional quality of orange fruits (see **Supplemental Table 5**). Transformation methods already exist for all of these species (Peña *et al.*, 1995; Ghorbel *et al.*, 2000; Iocco *et al.*, 2001). Genetic manipulation of photosynthesis would be an extremely promising approach in orange, due to the large yield improvements and additional improvements to nutritional quality.

1.4 Enhancing photosynthesis by manipulating photorespiration

As previously noted, increased atmospheric [CO_2] increases RuBisCO rates for CO_2 fixation and decreases the rate of photorespiration (Sage *et al.*, 2008). Given that *e*[CO_2] results in a down-regulation of photorespiration and prevents the accumulation of toxic intermediates that can inhibit the CBC, the predominant thought was that the down-regulation of photorespiration by genetic engineering would lead to improved productivity (Somerville and Ogren, 1982). However, knockdown of the H-protein of the glycine cleavage system (GCS, see Fig. 1.1) to ~50 % WT activity in barley was reported to inhibit photosynthetic efficiency and growth in conditions favourable to photorespiration (i.e. low atmospheric [CO_2] and high light) (Wingler *et al.*, 1997) and in rice knockdown of H-protein was shown to produce a lethal phenotype in ambient atmospheric [CO_2] (Zhou *et al.*, 2013). Knockdown of the rice H-protein transcript Os10g37180 was sufficient to inhibit photosynthetic efficiency and growth by up to 60 % but did not cause a lethal phenotype in ambient atmospheric [CO_2] (Lin *et al.*, 2016; Giuliani *et al.*, 2019). Reductions in the quantity of P-protein, which has also been shown to occur when H-protein is knocked down (Wingler *et al.*, 1997), to 30 – 40 % of WT activity in potato resulted in early senescence and a yellowing phenotype 5-6 weeks after germination and greatly reduced carbon assimilation (Heineke *et al.*, 2001) (for further review of these early studies, see Bauwe and Kolukisaoglu, (2003)). These negative effects of slowing down or inhibiting photorespiration are likely due to the creation of a metabolic dead end and the accumulation of toxic intermediates (South *et al.*, 2018). A follow up approach has revealed that increasing photorespiratory rates improves photosynthetic rates; a body of work examining the genetic manipulation of photorespiration is explored below.

1.4.1. Manipulation of photorespiration has identified target genes from the glycine cleavage system

The photorespiratory pathway recycles 2-PG, produced in the oxygenase reaction of RuBisCO, into 3-PGA that is then able to re-enter the CBC (**Fig. 1.1**). This pathway requires reactions that take place in the chloroplast, peroxisome, mitochondria and the cytosol and facilitates the recovery of approximately 75% of the carbon, with the remaining 25% being released as CO_2 in the mitochondria (Bauwe and Kolukisaoglu, 2003; Peterhansel *et al.*, 2010).

Manipulations to improve flux through the photorespiratory pathway have primarily examined the mitochondrial glycine cleavage system (GCS). The GCS is formed of 4 component parts – 2 enzymes that perform the reaction (P-protein, EC 1.4.4.2, and T-protein, EC 2.1.2.10), a protein that acts as a substrate shuttle (H-protein) and an enzyme that restores the H-protein coenzyme (L-protein, EC 1.8.1.4) (see **Fig. 1.1**) (Oliver *et al.*, 1990).

Timm et al., (2012) demonstrated that the up-regulation of the photorespiratory pathway could increase photosynthetic efficiency, possibly due to the reduction in quantity of photorespiratory intermediates that could negatively regulate CBC flux (such as glycine and glyoxylate).Since then, studies have been published examining overexpression of the T-protein, the L-protein and the H-protein of the GCS (see **Fig. 1.1; Supplemental Table 8**). Of these, T-protein overexpression was found to be unsuccessful in improving any photosynthetic parameters or increasing yield when expressed in the mesophyll of Arabidopsis (Timm *et al.*, 2018). L-protein overexpression in the mesophyll of soil-grown Arabidopsis resulted in a 19 % – 47 % increase in leaf dry weight compared to WT at 10 weeks under controlled, $a[CO_2]$ conditions with increased photorespiratory flux reported (Timm *et al.*, 2015). Arabidopsis overexpressing H-

protein in mesophyll tissue and grown in the same conditions as the previous study had an increase of dry weight of up to 33 % compared to WT (Timm *et al.*, 2012). Further work on H-protein by Lopez-Calcagno et al., (2018) found that constitutive H-protein overexpression in tobacco significantly inhibited growth, with transgenic leaf area only reaching 50 % of WT leaf area. This is in contrast to the result obtained by overexpressing H-protein in a tissue specific manor where plants had an up to 26 % increase in leaf area compared to WT. These findings demonstrate that constitutive expression of H-protein can be detrimental to plant growth. Additionally, shoot biomass gains of 26 % – 47 % and 13 % – 38 % were reported for the transgenic lines with tissue specific expression in field and greenhouse conditions respectively, demonstrating that H-protein overexpression can improve photosynthesis and yield in controlled and dynamic environments (Lopez-Calcagno *et al.*, 2018).

Increasing flux through the photorespiratory pathway has been hypothesised to prevent the build-up of toxic intermediates that inhibit the CBC. For example, it has been reported that 2-PG inhibits triose-phosphate isomerase (TPI) in pea (Anderson, 1971) and PRK in spinach (Kelly and Latzko, 1976), two CBC enzymes, which play important roles in the CBC and starch synthesis (see South et al., 2018 for review). More recent work in Arabidopsis shows that 2-PG inhibits the CBC enzymes TPI and SBPase inhibiting the formation of starch (Flügel *et al.*, 2017). Moreover, the accumulation of the photorespiratory intermediate glyoxylate has been shown to inhibit RuBisCO activation (Campbell and Ogren, 1990; Chastain and Ogren, 1989; Häusler *et al.*, 1996). Increasing photorespiration depletes these intermediates and it has been suggested that this effect reduces CBC inhibition (Timm *et al.*, 2015).

In addition to targeting the GCS, other authors have looked at the role of serine hydroxymethyltransferase (SHMT) in photorespiratory flux (**Fig. 1.1**). SHMT, together with the GCS, is responsible for the interconversion of glycine and serine (Bauwe and

Kolukisaoglu, 2003). In potato, plants with a reduction in SHMT were shown to have lower photosynthetic activity (Bauwe *et al.*, 1999). Zhang et al. (2019) demonstrated that the over-expression of SMHT in poplar resulted in an increase in photosynthesis, an increase in biomass and a 7 % – 8 % increase in cell wall glucose (**Supplemental Table 8**).

1.4.2. Up-regulation of photorespiration increases biomass yield but does not impact seed yield

Changes to photorespiration brought about by cultivation at high CO_2 or genetic manipulation of glycine decarboxylation clearly impact on growth rates and yield. However, work carried out in Arabidopsis shows that these modifications are far more complex than they originally appear. Firstly, down-regulation of photorespiration in *e*[CO_2] is accompanied by an increase in carbon assimilation due to high availability of CO_2. However, up-regulation of photorespiration in Arabidopsis resulted in no significant increase in photosynthetic carbon assimilation in plants grown at *a*[CO_2], suggesting that increases in biomass observed may be due to a reduction in the accumulation of toxic intermediates, a reduction in photoinhibition, and/or greater nitrate assimilation. These plants showed an increase in biomass yield, however no increase in seed yield was evident (Simkin et al., 2017a) (see **Supplemental Table 8**). These results highlight that further studies are required to determine if manipulating glycine decarboxylation alone is sufficient to increase harvestable yield of grain crops. Recent work in tobacco has shown that increasing photorespiration can increase biomass yield in a leaf harvestable crop (Lopez-Calcagno *et al.*, 2018) however these authors did not report seed yield.

1.4.3. Re-engineering the photorespiratory pathway using non-native genes

In addition to specifically modifying the photorespiratory cycle by over-expressing key subunits involved in glycine decarboxylation, an alternate approach to modifying photorespiratory flux has come from bypassing the photorespiratory pathway entirely by introducing photorespiratory bypasses using non-native genes.

The first such bypass was inspired by the cyanobacterial glyoxylate oxidation pathway, which reduces glyoxylate to glycerate I in the chloroplast. The advantages of this bypass are twofold – firstly, this pathway uses less ATP than the endogenous pathway and, secondly, moves the release of CO_2 from the mitochondria to the chloroplast where it can be re-fixed by RuBisCO (Dalal *et al.*, 2015; Kebeish *et al.*, 2007; Nölke *et al.*, 2014). In greenhouse grown plants, these authors observed an increase in photosynthesis and biomass in transgenic Arabidopsis, potato and camelina.

A second, non-native pathway engineered into plants moves all the steps for glycolate metabolism into the chloroplast. This includes glycolate oxidase (normally expressed in the peroxisome) to covert glycolate to glyoxylate, malate synthase to convert glyoxylate to malate, and a catalase enzyme to remove hydrogen peroxide (a by-product of the conversion of glycolate to glyoxylate). This pathway increases the CO_2 concentration around RuBisCO, outcompeting the oxygenation reaction of the native photorespiratory pathway. Expression of this pathway in Arabidopsis was shown to increase growth rates (Maier *et al.*, 2012). Additionally, re-engineering of this pathway in tobacco to remove the requirement for catalase resulted in an increase in photosynthesis and an increase in productivity of 19 % – 37 % in replicated field trials (South *et al.*, 2019).

In combination with other manipulations, these photorespiratory bypasses have the potential to confer cumulative increases in biomass yield and enhanced photosynthetic efficiency by increasing available CO_2 in the chloroplast and preventing the accumulation of potentially toxic intermediates. The impact of these pathways on grain or fruit yield have yet to be explored.

1.5. Alternate methods for increasing photosynthetic rates and CO_2 uptake through genetic engineering

A large body of work has developed looking at alternative gene targets to those in the CBC and photorespiration for improving photosynthetic efficiency and yield. This work can broadly be divided into two groups: manipulation of electron transport and expression of membrane proteins.

1.5.1. Manipulation of photosynthetic electron transport can increase photosynthetic efficiency

The photosynthetic electron transport chain (ETC) moves electrons between photosystem I (PSI) and photosystem II (PSII), creating an electrochemical proton gradient required for the synthesis of ATP and generates reducing equivalent (NADPH) for use in the CBC (**Fig. 1.2**). If greater electron transfer can be achieved then greater energy and more reducing cofactors would be available for the CBC, increasing flux capacity and carbon assimilation, though this is not without its limitations (see 1.1.2). This could be attained through a metabolic engineering approach.

Efforts to constitutively express cytochrome c_6 from algal species in Arabidopsis and tobacco have been made due to its role in electron transport, similar to plastocyanin (PC), as an electron carrier between cytochrome b_6f (cyt b_6f) and the PSI reaction centre (**Fig. 1.2**). In cyanobacteria and green algae, cytochrome c_6 (cyt c_6) has been shown to replace PC as an electron carrier in response to copper deficiency (Merchant and Bogorad, 1987). Furthermore, in Arabidopsis, Chida et al., (2007) showed that cyt c_6 transfers electrons from the cyt b_6f complex to PSI faster than has been reported for the native PC in *vivo*. Accordingly, transgenic Arabidopsis lines

expressing cyt c_6 grew faster and flowered earlier than WT (**Supplemental Table 8**), with WT only matching transgenic biomass towards the end of its (slower) development (Chida *et al.*, 2007). In tobacco, cyt c_6 expression enhanced growth in the leaves of 60 day old transgenic plants and was attributed to an over 60% increase in net photosynthetic rates (Yadav *et al.*, 2018).

Over-expression of the Rieske iron-sulfur (FeS; PetC) protein, a component protein of the cyt b_6f complex, as a way to increase photosynthetic electron transport has also been investigated (**Supplemental Table 8**). In a study from Simkin et al., (2017b), constitutive overexpression of the complex resulted in a 29 % – 72 % increase in aerial biomass, a 51 % increase in seed yield in the most productive line and early onset inflorescence development compared to WT; transgenic plants also had a greater rate of electron transport and carbon assimilation accordingly (Simkin *et al.*, 2017b). Simkin et al., (2017b) also showed that increasing the accumulation of Rieske FeS protein resulted in a concomitant increase in cyt *f* and cyt b_6 (**Fig. 1.2**), indicating that over-expressing a single subunit can result in an increase in other subunits of the complex. The level of the core protein of PSI (PsaA) and two proteins which form the reaction centre of PSII (PsbA; PsbD) were all shown to be elevated in Rieske FeS ox lines (Simkin *et al.*, 2017b). More recently, constitutive over-expression of the *Brassica rapa* Rieske FeS protein in Arabidopsis was shown to increase photosynthesis and electron transport rates in overexpressing lines. An increase in biomass (~50 % – 60 %) and early inflorescence development was also observed (Zhang *et al.*, 2020).

Over-expression of Rieske FeS has also been performed in the C4 species *Setaria viridis*, with a 10 % increase in complex content corresponding to a 10 % increase in carbon assimilation rate at both $e[CO_2]$ and $a[CO_2]$ (Ermakova *et al.*, 2019). This demonstrates that targeting single elements of ETC complexes is a viable approach for

improving photosynthetic electron transport and photosynthesis in both C3 and C4 species.

Several studies have also explored ferrodoxin (Fd; **Fig. 1.2**), one of the final acceptors of electrons in the transport chain. Increased capacity for ferrodoxin to receive electrons would increase capacity for photochemical quenching at the start of the ETC and enable greater thioredoxin reduction (through the activity of ferredoxin-thioredoxin reductase) for greater activation of CBC enzymes via reduced thioredoxin. Constitutive overexpression of ferredoxin in tobacco did not increase photosynthetic efficiency and no change in yield was reported (Yamamoto *et al.*, 2006). In addition, constitutive overexpression of ferrodoxin-NADP(H) reductase (FNR; **Fig. 1.2**), the enzyme responsible for enabling transfer of electrons from ferrodoxin to $NADP^+$ at the end of the ETC, also did not result in any significant changes in photosynthetic parameters in tobacco and growth of transgenic plants compared to WT was found to be reduced by up to 19 % across all lighting regimens (**Supplemental Table 8**) (Rodriguez *et al.*, 2006). These results suggest that Fd and FNR are not limiting in the ETC and that, in the case of FNR, over-expression may be detrimental to growth. However, constitutive over-expression of a plant ferredoxin-like protein (PFLP) in rice (*Oryza sativa*) did result in increased seed yield and improved electron transport (**Supplemental Table 8**) (Chang *et al.*, 2017). The role of PFLP in plants is predicted to be similar to ferrodoxin, although no functional analysis has yet been done. It should be noted that over-expression of PFLP resulted in a 2- to 4-fold increase in the transcript levels of CBC enzymes SBPase and FBPase (see **Fig. 1.1**) in addition to observed increases in AGPase and sucrose phosphate synthase (SPS) (Chang *et al.*, 2017). These increases in transcript levels resulted in a corresponding increase in protein levels. At this time, it is not known if the observed increases in electron transport, photosynthetic efficiency (+30 %) and biomass are a direct consequence of the over-expression of PFLP

or a by-product of PFLP induced increases in CBC, AGPase and SPS enzyme levels. However, over-expressing a single target that results in the concomitant increase in other photosynthetically relevant enzymes and proteins, as also observed with Rieske FeS over-expression (see above), is a viable option for improving photosynthetic efficiency and yield.

1.5.2. Expression of membrane proteins has been shown to increase CO_2 assimilation rates

The cyanobacterial inorganic carbon transporter B (ictB) was first identified from a *Synechococcus* sp. PCC 7942 with deficient carbon uptake. This was found to be due to a mutation in the ictB gene and the protein was ascribed a role in inorganic carbon transport (Bonfil *et al.*, 1998). Interestingly, since the protein was named, it has been suggested that ictB is in fact not an active carbon transporter, as the bacterial homologue Slr1515 from *Synechocystis* 6803 was demonstrated to have no independent bicarbonate transport activity (Shibata *et al.*, 2002). While these results imply that ictB is not an active carbon transporter, it is apparent that it has an indirect role in carbon assimilation rates as observed in tobacco, for example, expressing ictB in the chloroplast (Simkin *et al.*, 2015). Expression of ictB has been reported to improve photosynthetic efficiency and yield in multiple species (**Supplemental Table 9**). Transgenic Arabidopsis and tobacco constitutively overexpressing ictB, targeted to the chloroplast, were found to have lower [CO_2] compensation points than WT (~6 µL/L less than WT and ~9 µL/L less than WT respectively), indicating greater photosynthetic efficiency, and a concomitant increase in yield was also reported (Lieman-Hurwitz *et al.*, 2003; Lieman-Hurwitz *et al.*, 2005). The expression of ictB in rice was reported to increase photosynthetic rate and increase plant tiller number by up to 120 % in controlled conditions (Yang *et al.*, 2008) and in tobacco ictB expression resulted in a 19 % increase in photosynthetic rates, a 71 % increase in biomass and a reduction in water use efficiency (Simkin *et al.*, 2015). In soybean, ictB expression improved carbon assimilation and aerial yields significantly in both field and greenhouse trials (including a 30 % increase in seed yield in ambient [CO_2] conditions in Illinois trial fields in 2011) (Hay *et al.*, 2017). In Nebraska field trials, no significant increases in seed yield or total dry mass were reported, reflecting how variable climate conditions may impact on the

effectiveness of genetic manipulation of photosynthesis in field grown crops. A similar trend to this was identified in maize expressing ictB – an overall increase in grain yield of 3.49 % was reported across four growing seasons, with up to a 9.4 % increase in grain yield at specific trial sites, however no significant increase in yield was reported for the 2018 summer season (Koester *et al.*, 2021). In spite of this, a strong body of evidence supports the usefulness of ictB expression for improving carbon assimilation and plant yields under both controlled and field conditions.

Photosynthesis is limited by the rate CO_2 diffuses into the chloroplast. RNA interference of the Aquaporin NtAQP1 in tobacco reduced the CO_2 permeability of the inner chloroplast membrane suggesting that at least some Aquaporins function to allow CO_2 to diffuse across chloroplast membranes (Uehlein *et al.*, 2008; Heckwolf *et al.*, 2011; Moshelion *et al.*, 2015). Furthermore, photosynthesis is enhanced in Aquaporin overexpressing lines (**Supplemental Table 9**) (Uehleln *et al.*, 2003; Hanba *et al.*, 2004; Flexas *et al.*, 2006; Kawase *et al.*, 2013). Rice plants over-expressing Aquaporin were shown to have a 14 % increase in CO_2 assimilation rate and a 27 % increase in stomatal conductance (g_s) (Hanba *et al.*, 2004) and transgenic tobacco plants showed a 48 % increase in photosynthetic rate, 52 % higher mesophyll conductance and enhanced growth under well-watered growth conditions (Kawase *et al.*, 2013). It is conceivable that ictB acts in a similar manner, facilitating CO_2 diffusion, and further work is required to test this hypothesis.

Aquaporins and other membrane proteins offer an avenue to improve CO_2 assimilation allowing plants over-expressing CBC enzymes to fully benefit from those manipulations. In addition, the role of Aquaporins in the control of stomatal aperture suggests that manipulating Aquaporin levels targeted to stomatal guard cells could be an avenue for improving stomatal function and that ictB could potentially play a role if specifically targeted to guard cells (see Lawson et al., (2014) and references therein). In

the case of green fruit, such as immature tomato, green seed and embryos, a fully functional CBC, in the absence of stomata, appears to function to recover respiratory CO_2 released in the mitochondria and improves the movement of CO_2 from mitochondria to the chloroplast. By targeting ictB/aquaporins to the mitochondrial membrane, movement of this CO_2 could be increased and could significantly increase the rate of carbon recovery impacting on growth and nutritional quality (see Simkin et al., (2019) and references therein).

1.5.3. Overexpression of AGPase increases biomass and yield in a manner related, but separate, to increasing photosynthetic efficiency

AGPase performs the following reaction:

$$\text{Glucose-1-phosphate} + \text{ATP} \longrightarrow \text{ADPglucose} + PP_i,$$

where ATP = adenosine triphosphate, ADPglucose = adenosine diphosphate glucose and PP_i = pyrophosphate (Tuncel and Okita, 2013). This is the first step in committing photosynthetic carbon to starch synthesis. AGPase has previously been predicted as a rate-limiting enzyme in photosynthesis (Zhu *et al.*, 2007) and this has been confirmed via overexpression of AGPase showing increases to photosynthesis and yield in a range of plant species grown in controlled conditions (see **Table 1.2**). Many of these studies use the mutant *E. coli* gene *glgC16*, since a D336G mutation in an allosterically inhibitory site enables the expressed protein to have higher activity *in planta* (Stark *et al.*, 1992). AGPase genes from other organisms have also been used effectively however, only work from Meyer et al. (2007) has demonstrated this overexpression increasing photosynthesis and yield in the field. AGPase presents particular interest in strawberry due to hypothesised sink-limited growth of fruits in photosynthetically favourable conditions (Balasooriya *et al.*, 2019). Since AGPase could encourage greater photosynthetic carbon to be forced from the source leaf to the sink fruit, this potential limitation could be overcome.

Table 1.2: Overexpression of genes coding for AGPase subunits in a range of crops and the subsequent effects of that overexpression.

Plant	Gene(s)	Expression	Effect on yield	Reference
Maize	*glgC16* *E. coli*	Endosperm (sink)	13 – 25 % increase in seed weight. No change in number of seeds.	(Wang *et al.*, 2007)
	Bt2, Sh2 Maize	Endosperm (sink)	15 % increase in grain weight. ~10 % increase in seed starch content.	(Li *et al.*, 2011)
Wheat	*Sh2r6hs* Maize	Endosperm (sink)	338 % increase in seed weight and 31 % increase in biomass in controlled conditions. Similar increase achieved using promoters with different expression activities. Seed weight increases vary with field trial locations and do not occur in sub or non-optimal conditions. 3 – 7 % increase in CO_2 assimilation in high light at 5 days before flowering and up to 14 days after flowering.	(Smidansky *et al.*, 2002; Meyer *et al.*, 2007; Meyer *et al.*, 2004; Smidansky *et al.*, 2007)
	TaLSU1 Wheat	Endosperm (sink)	5.2 – 9.1 % increase in grain weight and 7.2 – 9.6 % increase	(Kang *et al.*, 2013)

			in starch weight for lines with highest AGPase activity.	
Rice	*UpReg-1* Potato	Chloroplast (source)	12 – 39 % increase in grain weight.	(Gibson *et al.*, 2011)
	glgC-TM *E. coli*	Cytoplasm	Up to 24 % increase in total grain weight.	(Nagai *et al.*, 2009a; Sakulsingharoj *et al.*, 2004)
	glgC-TM *E. coli*	Amyloplast	No significant effect on grain yield or biomass.	(Sakulsingharoj *et al.*, 2004)
	Sh2r6hs Maize	Endosperm (sink)	> 20 % increase in seed weight and plant biomass. No change in seed starch content.	(Smidansky *et al.*, 2003)
Cassava	*glgC16* *E. coli*	Tuber (sink)	Up to 2.6 fold increase in tuber biomass. Plants with largest tuber biomass increase also had increase in aerial biomass.	(Ihemere *et al.*, 2006)
Potato	*glgC16* *E. coli*	Tuber (sink)	Up to 60 % increase in tuberous starch.	(Stark *et al.*, 1992)
	glgC *E. coli*	Tuber (sink)	No significant change in tuberous starch.	(Stark *et al.*, 1992)
Tomato	*glgC* *E. coli*	Chloroplast (source)	No significant changes in phenotype. Non-significant 11.2 % average increase in leaf starch.	(Stark *et al.*, 1992)

1.6 Multigene manipulation of photosynthetic processes increases biomass

Recently a number of studies have looked at overexpressing several of these target genes in concert, a technique known as gene stacking. This is in an effort to further amplify the effects of single gene transformations to realise even greater photosynthetic efficiency and yield returns. This has so far resulted in vast yield increases, far beyond that achieved by single transformations. A summary of these single effects can be seen in **Table 1.1** and gene stacking effects in **Table 1.3**.

1.6.1. Simultaneous manipulation of Calvin-Benson-Bassham cycle and photorespiration can have a synergistic effect on yield in controlled conditions

Co-overexpression of SBPase and FBPA has been investigated in tobacco and Arabidopsis alongside expression of ictB and the over-expression of GCS H-protein (**Fig. 1.1**). In the former study, constitutive overexpression of the three genes in tobacco (SBPase, FBPA, IctB) was used and a 103 % increase in total dry weight, formed largely of gains to aerial tissue mass, was reported for the triple-expressing transgenic line relative to WT plants (Simkin *et al.*, 2015). The study in Arabidopsis used leaf tissue specific expression for all three genes (SBPase, FBPA, GCS H-protein). After 38 days of growth in low light, triple expressing lines had a 70 % increase in dry weight compared to the WT and null controls. In high light conditions, a 46 % increase in final vegetative biomass was reported (Simkin *et al.*, 2017a). This was accompanied by increased photosynthetic parameters in both studies. Curiously in both studies, the highly productive triple-expressing lines didn't exhibit the greatest increases in

photosynthesis out of all lines tested under normal atmospheric conditions, despite having the greatest changes in growth and biomass compared to WT. This may suggest that interactions between overexpressed genes have an enhancement effect that is not measurable by photosynthetic capacity alone or that photosynthetic enhancement occurs at times of day that haven't been studied, such as during periods of shade or low light at dawn and dusk where transgenic lines with specific manipulations overcome limitations present in other lines. Further study is needed to identify these mechanisms.

As previously noted (see above), increasing photorespiration in Arabidopsis led to an increase in biomass yield and no increase in seed yield (Simkin *et al.*, 2017a; Kebeish *et al.*, 2007; Timm *et al.*, 2012). This is in contrast to plants over-expressing the Arabidopsis SBPase (AtSBPase) and FBPA (AtFBPA), both of which showed increases in both biomass and seed yield. Combining the over-expression of AtSBPase and AtFBPA (increasing CBC activity) with increases in photorespiration (by over-expression of the GDC-H protein), results in a cumulative increase in seed yield, suggesting that increasing photorespiration (resulting in a decrease in toxic intermediates) can increase seed yield when combined with an increase in carbon assimilation rates due to increased CBC activity (Simkin et al., 2017a). It may possible to draw parallels here with a decrease in photorespiration (resulting in a decrease in toxic intermediates) and increases in carbon assimilation in *e*[CO_2] environments resulting in higher fruit yields.

1.6.2. Manipulation of carbon assimilation results in increased biomass in the field

An evaluation of the function of the cyanobacterial enzymes SBPase (cySBPase), FBPase (cyFBPase) and the bifunctional FBP/SBPase (FS Bif) in tobacco has also been carried out. These results can be considered a multi-targeted approach, as the cyanobacterial FBP/SBPase carries out both SBPase and FBPase enzymatic reactions (**Fig. 1.1**). Tobacco expressing cySBPase showed a 20 % increase in the rate of photosynthetic CO_2 fixation and a 50 % increase in final biomass. Plants expressing cyFBPase displayed a 15 % increase in CO_2 fixation rates and a 30 % increase in biomass compared to controls (**Table 1.3**). Tobacco plants producing the FS Bif enzyme showed a 70 % increase in FBPase activity and a 130 % increase in SBPase activity, enhancing photosynthetic activity by 20% and increasing biomass by 40 % – 50 % after 18 weeks of growth (Miyagawa *et al.*, 2001). Moreover, the expression of FS Bif in lettuce increased photosynthetic activity by 30 % and fresh weight of 6 week old plants by 60 % (Ichikawa *et al.*, 2010). Both studies were carried out in controlled environment conditions.

Further research in tobacco has recently been reported examining how expression of FS Bif alongside cytochrome c_6 can increase photosynthesis and yield in field conditions. A 27 % increase in yield at the flowering stage was reported for field grown tobacco with co-expression of FS Bif and cytochrome c_6 while no increase was seen for single expressing lines, indicating that genetic manipulation of photosynthesis through gene stacking can lead to an increase in final harvestable yield for field grown plants (López-Calcagno *et al.*, 2020). Understanding how plants with genetically improved photosynthesis respond in the field is vital to better understand how the manipulations tested in these studies could be applied to agricultural systems, such as fruiting crops.

The response of a crop with multigene manipulations to growth in field conditions has been tested in a study by Gong et al., (2015). Rice lines expressing only FS Bif or ictB alone displayed no significant changes in yield compared to WT. However, in the double expressing line, improved photosynthetic parameters and increased yield (through increased tiller number) compared to WT were reported (Gong *et al.*, 2015). This demonstrates that gene stacking has potential to improve photosynthesis and yield of crops species in addition to model species (see above) grown in the field and may be a more effective method for improving these parameters than overexpression of single gene targets (**Table 1.1**). It must be noted that FS Bif expression did not increase yield in these studies when expressed individually, showing that not all multi-targeted approaches translate to greater yield gains in the field. However, FS Bif expression in concert with other genes did result in increased photosynthesis and yield, demonstrating its viability in gene stacking photosynthetic targets.

These studies are very important in signposting the way forward for research into enhancing photosynthesis. Improving multiple targets simultaneously can produce much greater results compared to single gene modifications. As such, it is important that this field continues to examine which combinations of genes can be stacked to produce the largest biomass and growth returns and begin to integrate this with alternative methods for improving photosynthetic efficiency to study further enhancement effects. This could be achieved by creating species-specific models of the CBC accounting for experimentally observed differences in flux or by trialling new combinations of well-studied single gene manipulations. An example that could have direct consequences in strawberry would be engineering low-specificity, highly catalytic RuBisCO in upper canopy leaves and vice-versa in lower canopy leaves to mitigate effects of canopy shading. This would also require greater research into promoters activated by the red/far-red light ratio to ensure precision engineering of foliar

photosynthesis in this manner, demonstrating how promoter research is an essential area of study for optimised photosynthetic efficiency (Ort *et al.*, 2015). This multigene targeting approach is also just the tip of the iceberg. Many other studies have identified targets for manipulation that increase yield such as AGPase (Nagai *et al.*, 2009b; Li *et al.*, 2011), geranylgeranyl pyrophosphate synthase (Tata *et al.*, 2016) and the manipulation of nitrate mobilisation in plants (K., E., Chen *et al.*, 2020) to name a few. Furthermore, a recent review has delved into the co-manipulation of photosynthetic and vitamin metabolism targets for the generation of high yielding multivitamin crops for addressing 'hunger' and 'hidden hunger' in at risk populations (see Simkin (2019) and refs therein). A broad study of combining these manipulations is needed to assess the synergistic effect of these works on yield and quality.

Manipulation	Plant	Transgene(s) Expressed	Biomass and Yield	Reference
CBC and Photorespiration	Arabidopsis Col-0	SBPase - -	42% increase in dry weight. 53% increase in seed yield under LL (39% under HL).	(Simkin *et al.*, 2015)
		- FBPA -	32% increase in dry weight. 35% increase in seed yield under LL (36% under HL).	
		SBPase FBPA -	41% increase in dry weight. 49% increase in seed yield under LL (20% under HL).	
		- - GCSH	50% increase in dry weight. 0% increase in seed yield under LL (0% under HL).	
		SBPase FBPA GCSH	71% increase in dry weight. 42% increase in seed yield under LL (62% under HL).	
CBC and CO_2 transport	Tobacco cv Samsun	SBPase - -	30-34% increase in dry weight under HL (52% under LL).	(Simkin *et al.*, 2017)
		- - ictB	71% increase in dry weight under HL.	
		SBPase - ictB	92% increase in dry weight under HL (76% under LL).	
		SBPase FBPA -	62% increase in dry weight under HL (54% under LL).	
		SBPase FBPA ictB	103% increase in dry weight under HL (79% under LL).	
CBC and CO_2 transport	Rice	FS Bif - -	No increases in biomass were observed.	(Gong *et al.*, 2015)
		- - ictB	No increases in biomass were observed.	
		FS Bif - ictB	An increase in biomass demonstrating the synergistic effect.	
		SBPase - -	9% increase in aerial biomass in glasshouse.	(López-Calcagno

CBC and Electron Transport	Tobacco cv Samsun	SBPase	Cyt c_6	-	32% increase in aerial biomass in glasshouse.	et al., 2020) (Lopez-calcagno et al., 2018)
	Tobacco cv Petit Havana	FS Bif	-	-	35% increase in aerial biomass in glasshouse, 25% in field at 39 days with no difference to control at flowering stage.	
		-	Cyt c_6	-	44% increase in aerial biomass in glasshouse, 36% in field at 39 days with no difference to control at flowering stage.	
		FS Bif	Cyt c_6	-	52% increase in aerial biomass in glasshouse, 27% in field at flowering stage.	
CBC	Tobacco	FS Bif	-	-	50 % increase in biomass for 18-week old plants grown in hydroponic growth chambers.	(Miyagawa et al., 2001)
	Lettuce	FS Bif	-	-	60 % increase in fresh weight of 6-week old plants grown in hydroponic growth chambers.	(Ichikawa et al., 2010)
	Soybean	FS Bif	-	-	Up to 31 % reduction in seed yield for WT plants grown in elevated [CO_2] = 600 ppm and elevated temperature = ambient + 3.5 °C with no reduction for transgenic plants. Up to 9 % lower harvest index in WT plants compared to transgenic lines grown at ambient conditions. Plants grown in field.	(Köhler et al., 2017)

Table 1.3: Description provided below

Table 1.3: Summary of multigene manipulations to increase photosynthetic efficiency. The co-over-expression of Calvin–Benson Cycle, Photorespiration, Photosynthetic Electron Transport

and CO2 transport proteins and their biological outcomes and shown. (Arabidopsis; LL; Low Light = 130 mol/m−2/s−1 and HL; High Light = 390 mol/m−2/s−1. Tobacco LL = 200–350 mol/m−2/s−1; HL = 600–1400 mol/m−2/s−1). Increases in dry weight and seed yield are indicated. Sedoheptulose-1,7-bisphosphatase (SBPase); fructose-1,6-bisphosphate aldolase (FBPA); Bifunctional FB/SBPase (FS Bif); Glycine decarboxylase H protein (GCS H) Cytochrome c6 (Cyt c6); inorganic carbon transporter B (ictB) and the cyanobacterial enzymes SBPase (cySBPase), FBPase (cyFBPase) and the bifunctional FBP/SBPase (FS Bif).

1.7. Does increasing carbon assimilation increase environmental tolerances?

Efforts to overexpress SBPase in rice under greenhouse conditions returned biomass increases at high temperatures (Feng, Wang, *et al.*, 2007) and under salt stress (Feng, Han, *et al.*, 2007) compared to untransformed controls. These transformations did not translate to biomass increases under unstressed conditions in both cases (25 °C), however these studies highlight how photosynthesis can be made more tolerant, operating under conditions of plant stress.

As noted earlier, Gray et al., (2016) demonstrated in soybean that elevated CO_2 was insufficient to protect yields from drought conditions of elevated temperature and reduced water availability. Work from Köhler et al., (2017) studied the effects of FS Bif expression on field grown soybean and found that at both *a*[CO_2] and *e*[CO_2] at ambient temperature, a significant increase in seed yield for transgenic versus WT plants was present by the 3rd year of trials. In *e*[CO_2] and elevated temperature (+3.5°C), overexpression of FS Bif was reported to maintain seed yield compared to reduced yield for the WT, demonstrating that this transformation could be useful in designing crops for future climate conditions (Köhler *et al.*, 2017). Further investigation of how FS Bif transformed plants respond under water stress (i.e. in similar drought conditions to those used in Gray et al., 2016) could provide further insight into the interaction of multiple stresses and how they are potentially mitigated by elevated photosynthesis. Overall FS Bif expression can improve the yield of both model and crop plants and could be useful in mitigating yield losses from certain climate change scenarios (where irrigation is maintained) of increased temperature and elevated atmospheric [CO_2].

An alternative approach to improving crop tolerance to temperature concerns engineering of RuBisCO activase (RCA). RCA enables RuBisCO to enter an active

configuration but its high heat lability causes a loss of function (and thereby, limitation of carbon fixation) at high temperatures, including those theorised to be optimal for photosynthesis. The temperature at which RCA becomes limiting varies on a crop-by-crop basis (Qu *et al.*, 2023). Increasing the thermo-tolerance of RCA to reduce heat lability can improve photosynthetic performance at higher temperatures, since RuBisCO can be more readily activated for carbon fixation (Kurek *et al.*, 2007), but this can still be limited by reduced stomatal conductance to limit water evaporation under high temperatures (Hussain *et al.*, 2021). Alternatively, growing crops or cultivars that already have more thermostable RCA in warming areas could help buffer against loss of yields from rising temperatures without a need for engineering of photosynthesis.

These works highlights the potential for genetically manipulating CBC to future proof crop plants against expected changes in climate, where maintaining yield at current levels may be as important as increasing yield due to the adverse effects of a changing climate. Even so, it is conceivable, or even likely, that amplifying photosynthesis may not be sufficient to prevent yield losses. In such cases other avenues must be explored, such as improving soil nitrogen and nitrogen uptake strategies and fertilising soil with microorganisms both beneficial to growth and tolerant of changing temperatures (Soares *et al.*, 2019). An integrated response such as this may be necessary if climate change continues on its current course.

1.8. Over-expression of known enzymes can have unexpected outcomes

Unfortunately, overexpressing enzymes of the CBC to improve plant photosynthesis and yield is not as simple as it seems. The CBC enzyme plastid transketolase (TK) has been demonstrated to limit photosynthetic flux in cucumber – TK knockdown reduced photosynthetic parameters and inhibited growth – making this another potential target (Bi *et al.*, 2014). However, overexpression of TK has so far yielded unexpected results. In a study from Khozaei et al., (2015), constitutive TK overexpression in 5 week old tobacco plants resulted in a chlorotic, slow growth phenotype and no change in carbon assimilation rate was found; this was ultimately attributed to a vastly depleted thiamine pool that resulted from excess TK activity (Khozaei *et al.*, 2015). Contrary to this, overexpression of TK in rice was reported to effect no change in plant biomass or rate of carbon assimilation, nor was a chlorotic phenotype reported (Suzuki *et al.*, 2017). In addition, TK overexpression in cucumber gave no chlorotic phenotype and was reported to significantly increase plant biomass during early development due to an increased number of female flowers (Bi *et al.*, 2013). It is apparent that TK has widely variable effects on different plant species. To better understand this, further research is needed on how overexpressing different CBC enzymes differentially affects photosynthesis (and more broadly, plant primary metabolism) and how these differences can be manipulated for species-specific metabolic engineering.

1.9. Future directions

A wide range of target genes are available for expression and overexpression for improvement of photosynthesis, including targets from the Calvin-Benson-Bassham Cycle and other metabolic origins. Gene stacking of these targets into fruit crops could lead to large returns in fruit yield, quality and nutritional traits, in a species and cultivar specific manner, based on how these crops respond to growth in carbon-enriched atmospheres.

Elevated [CO_2] has previously been shown to increase Vitamin C content in sun-acclimated leaves of sour orange (Schwanz *et al.*, 1996). Wu et al. (2017) has shown that growing carrots in *e*[CO_2] also results in the accumulation Vitamin C due to changes in the expression levels of 12 biosynthetic genes, with similar results and a 1.09 – 3.91-fold increase in Vitamin C being found in celery petioles (Liu *et al.*, 2020). This increase in expression may be caused by feedback of CBC products regulating the electron transport chain (Rochaix, 2011), since redox state of the plastoquinone pool regulates expression of Vitamin C biosynthesis genes (Karpinski *et al.*, 1997). The observed increase in Vitamin C observed in tomato fruit and other crops grown at *e*[CO_2] suggest that increasing carbon uptake, either through manipulating growth conditions or genetically enhancing the ability of plants to take in carbon, can positively influence the Vitamin C content of foods. Vitamin C is well tolerated, an important dietary requirement, and often taken as a supplement. At high concentrations it has been used as a treatment of cancer, arteriosclerosis, and cardiovascular diseases (Du *et al.*, 2012; Leong and Oey, 2012; da Silva Dias, 2014). These results suggest that enhancing photosynthesis could contribute to an uplift in Vitamin C in 'engineered' crops improving their nutritional value. However, other studies have shown that growing barley at *e*[CO_2] resulted in a significant decrease in Vitamin C content (Robinson and

Sicher, 2004) highlighting the species–species response differences to *e*[CO_2] and differences that will likely be observed in engineered fruit crops (see Fenech et al. (2019) and references therein).

In tomato fruit, the concentration of lycopene and β-carotene (pro-vitamin A) have been shown to increase in response to *e*[CO_2] (per unit dry weight). Lycopene is an important phytonutrient, is sold commercially as a dietary supplement, and has been reported to possess anti-cancer properties and can improve cardiovascular health (see Story et al. (2010) and references therein). Furthermore, the increases in total carotenoids observed by Rangaswamy et al. (2021) imply that there would be an equal or higher increase in β-carotene (not measured) in these tomato fruit. Increases in β-carotene in tomato fruit under *e*[CO_2] were reported by Zhang et al. (2014). β-carotene is the precursor for Vitamin A, also known as retinol. Vitamin A is an essential micronutrient playing important roles in growth and development, vision (Rando, 1990) and the immune system (West *et al.*, 1991). More than a third of all preschool children and a significant number of pregnant women around the world are affected by Vitamin A deficiency, increasing the risk of night blindness and miscarriage (WHO, 2019; WHO, 2005). Importantly, most people suffering from a deficiency in Vitamin A show no clinical symptoms resulting in a phenomenon termed 'Hidden Hunger' (Hodge, 2016). Production of crops with increased Vitamin A is therefore an important target for improving the diet and health of these at-risk groups; enhanced uptake of carbon may be a useful approach to achieve this.

The data presented in this chapter suggest that increasing CO_2 uptake can result in improved yields and a more nutritious crop with improved flavour qualities. This work also suggests that there is scope to improve these attributes through the genetic manipulation of photosynthesis providing a better tasting, more nutritious product. However, it must be noted that not all work has shown that growing crops in *e*[CO_2] has

had a beneficial impact on quality. For example, although increases in carotenoid content were observed in tomato grown at 550 ppm, a decrease was observed in these fruits when grown at 700 ppm. In the non-fruiting crop soybean, seed yield increased in *e*[CO_2], however these authors reported a reduction in seed oil concentrations and seed protein levels (Cober and Morrison, 2019). Furthermore, a meta-analysis of C3 plants and legumes found a reduction in zinc and iron (and in non-legumes a reduction in protein) when plants were grown under *e*[CO_2] (see Myers et al., (2014)). In 2018, Zhu et al., (2018) confirmed these results, and moreover demonstrated that rice grown under *e*[CO_2] accumulated reduced concentrations of vitamins B1, B2, B5, and B9. With global increases in atmospheric [CO_2] from 417 ppm to 550 ppm by 2050 (IPCC, 2007a; Le Quéré *et al.*, 2009) and increased temperature and rainfall, these results demonstrate that climate change could significantly impact the nutritional quality of our crops. Future research needs to address how and why different crop/fruit crop species respond differently to growth in enriched carbon atmospheres. This will pave the way forward for precision genetic engineering of photosynthesis; exploring both overexpression and knockdown of genes in concert and in a species-specific manner.

The work presented here also suggest that increasing CO_2 uptake could have other benefits. For example, in melon (*Cucumis melo*), growing plants in *e*[CO_2] has been shown to mitigate yield losses from increased salinity (Mavrogianopoulos *et al.*, 1999). Interestingly, a parallel exists between photosynthetically genetically modified crops and increased tolerance to salinity. In Arabidopsis, over-expression of SBPase, which enhances CO_2 assimilation rates, enhances salt tolerance by increasing the sugar and chlorophyll content (Chen *et al.*, 2020). It could be hypothesised that growing plants under *e*[CO_2] would similarly increase sugar and chlorophyll content triggering a similar mechanism of salt tolerance. It would be interesting to explore on a greater scale if increased CO_2 assimilation rates, through atmospheric manipulation or genetic

modification, can have a positive impact on crop resistance to high salt environments and other abiotic stresses. There is currently evidence that over-expressing photosynthetic genes can increase tolerance to chilling stress (SBPase; Ding et al., (2016)) and prevent yield loss under high temperature (FS Bif; Köhler et al., (2017)). Köhler et al. (2017) concluded that the manipulation of CO_2 uptake could mitigate against the effects of global increases in temperature under $e[CO_2]$. This may be deemed especially important given the expected impact of global climate change.

1.10. Concluding remarks

Overall, fruiting crops appear to benefit from growth in carbon enriched atmospheres, though this may come with an improvement in some quality traits at the expense of others – therefore, it will be necessary to determine the optimal growing environment (i.e. CO_2 concentrations and temperature) to obtain high yielding and high-quality produce. Genetic manipulation of photosynthesis would similarly increase CO_2 uptake and is predicted to generate large improvements in fruit yield, similar to the effects of carbon-enriched growth. If a genetics approach is taken, improving photosynthesis may allow plants to take full advantage of the atmospheric [CO_2] available at ambient levels. Such manipulations, in some cases, may allow crops to be grown outdoors at ambient [CO_2] rather than in expensive greenhouse environments where [CO_2] needs to be elevated, placing a financial burden on the producer. It will also be necessary to evaluate the impact of these genetic modifications on fruit quality.

Genetic transformation protocols of several fruiting crop species discussed in this review have been reported (see above), although chili pepper transformation is difficult due to its poor regenerative capacity *in vitro* (Ochoa-Alejo and Ramirez-Malagon, 2001). This means that genetic manipulation of photosynthesis in fruiting crops is achievable with known methods, increasing the viability of this approach. The capacity for simultaneous improvement of a range of both yield and quality traits to generate a new generation of 'high yielding multivitamin crops' for addressing 'hunger' and 'hidden hunger' in at risk populations has previously been postulated (see Simkin, (2019) and references therein). In conclusion, there exists a great degree of potential for genetic manipulation of photosynthesis in fruiting crops, which could lead to improvements in multiple traits which are difficult to increase simultaneously through traditional breeding methods.

Chapter 2 – Materials and Methods

2.1 – Introduction

This chapter describes the general materials and methods used throughout the thesis. Where necessary, specific materials and methods are described in the appropriate thesis chapters.

2.2 – Experimental Field Work Protocols

2.2.1 Plant Growth Conditions and Experimental Setup

An 8.5 m x 50 m polytunnel was set up along the north-south axis at NIAB EMR (51.29 °N, 0.45 °E) and covered with Classic Clear LUV plastic sheeting (Berry BPI). Everbearing strawberries cv. Malling Centenary were planted in one metre coir bags (CocoGreen) at eight plants per bag. Six metal rows were erected in the polytunnel for table top production and 48 planted coir bags placed on each row for a total of 384 plants per row. Plants were irrigated and sprayed as directed by the Berry Gardens Growers Agronomy Team under the direction of Mr Justas Baroniunas. Harvesting of the entire tunnel by pickers occurred twice a week on Tuesdays and Fridays commencing on 15/06/21 and finishing on 05/11/21. Harvesting for yield measurements occurred twice a week on Mondays and Thursdays, commencing on 14/06/21 and finishing on 04/11/21. Plants used for this study were the eight plants grown in the central bag of segments 7 – 10 (see **Fig. 1** for a graphical visualisation), giving 32 plants per row for study.

2.2.2 Photosynthetic Data Collection

Early season measurements were taken during the first flush from 12/06/21 – 16/06/21. Mid-season measurements were taken during the second flush from 27/07/21 – 10/08/21. Measurements were taken using the LCpro-SD iFL Portable Photosynthesis System (ADC Bioscientific Ltd., see **Fig. 2.1**). Measurements were taken once during a morning session beginning at least half an hour after sun up (from ~6.00 AM onwards) and during an afternoon session ending before sunset (by ~8.00 PM). The photosynthetic characteristics of the strawberry leaves were tested by point measurements and light response curves. Tested leaves were well exposed to sunlight (i.e. not obscured by the upper canopy), were young, fully

expanded, non-senescent and showed no signs of disease, pests or damage. Measurements were taken in the following pattern:

1. Four point measurements per row;
2. Two light response curves, one for two of the rows selected via a randomised block design;
3. Four point measurements per row;
4. Two light response curves, one for each of the two rows not yet tested;
5. Four point measurements per row.

Point measurements were performed by exposing the leaf to saturating light (set at 1500 µmol m^{-2} s^{-1}) with no other environmental conditions controlled (i.e. carbon dioxide, water vapour, temperature) to better match the natural environment of the polytunnel. The leaf was clamped into the chamber and held for ~one minute. This was to allow the infrared gas analyser (IRGA) to take an accurate reading of changes to the chemical composition of the air at a steady state of photosynthesis. Point measurements were performed four times a row, three times per session across the morning and afternoon. This coincided to measurements being taken at 6.30 AM, 8.30 AM, 11.00 AM, 3.30 PM, 5.30 PM and 7.30 PM. Plants used for measurements were selected at random from one of the four segments, with different segments being used at each block of point measurements.

Light response curves were taken using a programme of decreasing light intensities with measurements taken at one minute intervals with each light intensity held for two minutes. This programme is shown in **Table 2.1**. Two light response curves were taken between each set of point measurements in the morning and afternoon session. This meant each row was measured once during the morning and the afternoon of any given day. A randomised block design was used to ensure rows were measured at different times in each morning or afternoon session, to account for differences between measurements taken at different times during the session.

Over the course of several days of measurements, each row was measured at least once during the early morning, early-mid morning, late-mid morning and late morning for the morning session and the same for the afternoon session. This was to minimise variation in the results of the light response curves that could have been caused by time of day.

Both point measurements and light response curves recorded carbon assimilation (*A*), leaf evaporation of water vapour (*E*), leaf internal carbon dioxide concentration (*Ci*) and stomatal conductance (*gs*). Intrinsic water use efficiency (*WUEi*) was calculated through the following formula:

$$WUEi = A/gs$$

Step Number	Light Intensity (µmol m^{-2} s^{-1})	Hold Time (mins)
1	1500	25
2	1300	2
3	1100	2
4	900	2
5	700	2
6	550	2
7	400	2
8	250	2
9	150	2
10	100	2
11	50	2
12	0	2

Table 2.1: Stepwise description of light response curve programme.

2.2.3 Fruit harvesting

Ripe fruits were harvested twice weekly on Mondays and Thursdays. Class 1, Class 2 and waste yields were recorded alongside fruit number. Class 1 fruit were defined as having a diameter greater than 25 mm, < 5 % white colouration with no dirt or deformities. Class 2 fruit were defined as failing to meet all the specifications of Class 1 fruit while still having a diameter greater than 18 mm, < 10 % white colouration and at most minor deformities. Waste fruit were defined as fruits that did not meet all the specifications for Class 1 or Class 2 fruit. The row and plant from which each fruit originated was also recorded. Fruit collected here were added to fruit harvested by pickers from the rest of the polytunnel on Tuesdays and Fridays to determine total fruit yield for each whole row.

2.2.4 Leaf harvesting

Leaves tested during photosynthetic light curve measurements were harvested by cutting into 3 smaller segments, during which the midrib was removed, placed in 2 mL collection tubes and immersed immediately in liquid nitrogen to limit degradation of the RNA. Leaf samples were then stored at -80 °C until RNA extraction.

Fig. 2.1: Image of experimental setup for light curve measurements with the ADC machine.

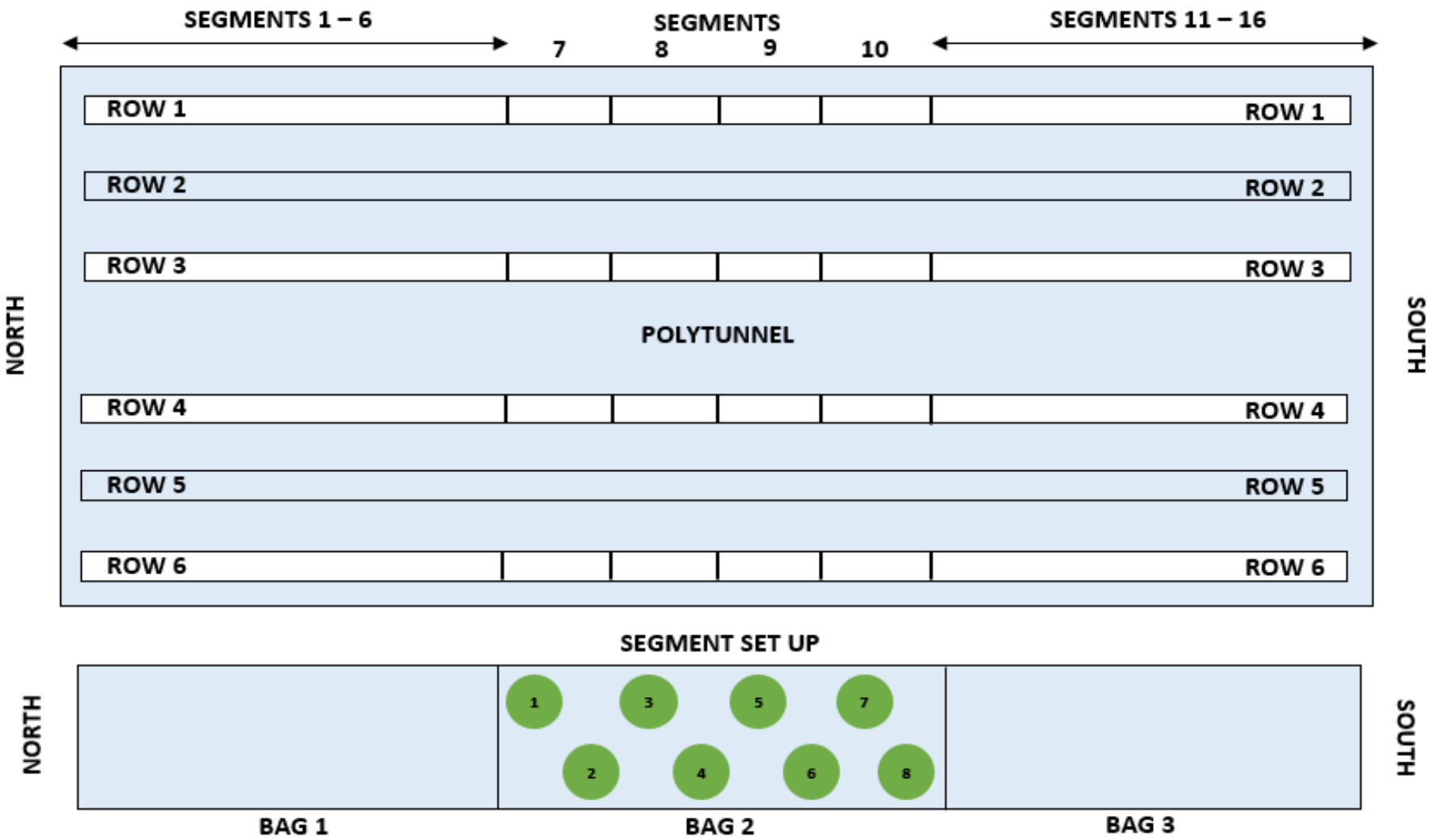

Fig. 2.2: Experimental setup of the polytunnel. Tested rows are highlighted in white. Green dots represent planting pattern of individual strawberry plants. 8 plants per bag, 3 bags per segment. Central bag of each segment used for photosynthetic measurements and fruit collection to avoid interaction with metal structures between segments.

2.3 – Generation of Plasmids

2.3.1 cDNA synthesis

Arabidopsis thaliana (*A. thaliana*) was grown at 120 µmol m^{-2} s^{-1} with a 16/8 photoperiod to four weeks old. RNA was extracted from young developing leaves as directed by NucleoSpin RNA Kit (Macherey-Nagel). Gene sequences for *A. thaliana* SBPase (AT3G55800) and AGPase large subunit APL1 (AT5G19220) were identified using NCBI Gene Blast and primers designed using NCBI Primer Blast. Primer sequences were designed as followed:

Primer	Direction	Sequence
SBPase	Forward	CACCATGGAGACCAGCATCGCGTGC
SBPase	Reverse	GTTTCTAAGCGGTAACTCCAATGG
AGPase	Forward	CACCATGGTGGTCTCTGCTGACTGC
AGPase	Reverse	CTTAAAAGTATCATATCACAACTCC

Table 2.2: Primer sequences for amplifying *Arabidopsis thaliana* genes of interest.

Synthesis and subsequent amplification of cDNAs was performed as directed by SuperScript III Reverse Transcriptase Kit (Invitrogen). Samples were purified as directed by NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). At each processing step, DNA was visualised against a 1 kb DNA ladder (NEB Biosystems) via gel electrophoresis using an agarose gel (0.75 %, Fisher Bioreagents) made with TAE buffer (50 x dilution, Fisher Bioreagents) stained with GelRed (10 µL L^{-1}, Biotium). Gels were ran for 30 minutes at 100 volts and then for further 10 minute intervals as needed to ensure full separation of DNA bands. This was done to confirm successful generation of the desired cDNAs.

2.3.2 Cloning of cDNAs into a vector

Purified cDNAs were cloned into the pENTR/D-TOPO vector as directed by pENTR/D-TOPO Cloning Kit (Invitrogen) (see **Fig. 2** for vector map).

2.3.3 Chemical transformation of Escherichia coli

Stock of *E. coli* (strain DHAα) stored in glycerol (25 %, Fisher Bioreagents) was thawed on ice. Cloned plasmid (3 µL) was added to bacteria and the mixture rested on ice for 5 minutes. Samples were then heated in a water bath at 42 °C for 45 seconds, followed by a 1 minute incubation on ice. High salt LB broth (250 mL, Melford) was added to cells. Cells plated on high salt LB plates with kanamycin (50 µg mL^{-1}, Duchefa Biochemie) and incubated at 37 °C overnight. Successful transformation was confirmed using colony PCR (see 2.3.4).

Comments for pENTR™/D-TOPO®
2580 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (c)
TOPO® recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO® recognition site 2: bases 689-693
*att*L2: bases 705-804
T7 Promoter/priming site: bases 821-840 (c)
M13 reverse priming site: bases 845-861
Kanamycin resistance gene: bases 974-1783
pUC origin: bases 1904-2577

(c) = complementary sequence

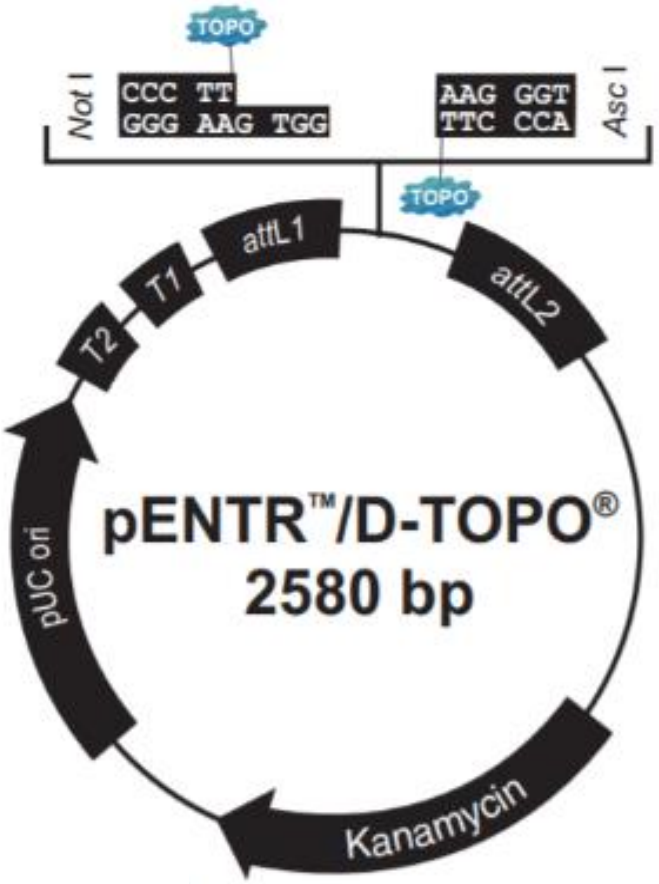

Fig. 2.3: Map of pENTR/D-TOPO vector with comments on component parts. Adapted from Invitrogen manual.

2.3.4 Colony PCR

Individual colonies of *E. coli* were collected with a sterile pipette tip and touched against a high salt LB (Melford) plate with kanamycin (50 µg mL^{-1}, Duchefa) before being added to a PCR mix (see master mix recipe in **Table 2.3**). Plate was incubated at 37 °C overnight, with successful colony growth indicating successful transformation. PCR was ran as follows:

1. 95 °C for 2 minutes;
2. 45 cycles of 95 °C for 30 seconds, followed by 55 °C for 30 seconds, followed by 72 °C for 1 minute 45 seconds;
3. 72 °C for 10 minutes; 15 °C until taken out of cycler.

PCR results were confirmed via gel electrophoresis using an agarose gel (0.75 %, Fisher Bioreagents) made with TAE buffer (50 x dilution, Fisher Bioreagents) stained with GelRed (10 µL L^{-1}, Biotium). DNA was compared against a 1 kb DNA ladder (NEB Biosystems).

Component[1]	**Volume – 1 sample (µL)**	**Volume – n samples (µL)**
Buffer	1.5	1.5n
dH_2O	12	12n + n
Forward Primer	1	$0.1n^2$
Reverse Primer	1	$0.1n^2$
dNTPs	0.3	0.3n
Taq polymerase	0.25	0.25n

Table 2.3: Master mix recipe for colony PCR. [1]Primers concentration at 10 µg mL^{-1}. [2]Minimum primer volume of 1 µL.

2.3.5 Golden Gate assembly

Constructs cloned via Golden Gate Assembly and expressing SBPase and AGPase individually under the control of constitutive and tissue specific promoters were donated by the University of Essex. Level 1 genetic elements and Level 2 destination vectors were also donated by the University of Essex. Plasmids constructed with both SBPase and AGPase (under tissue specific or constitutive control) were cloned via Golden Gate Assembly (Engler *et al.*, 2009; Engler *et al.*, 2008) using the modular cloning system. Each reaction was performed as follows:

Reagent	**Volume (µL)**
dH_2O	3.18
T4 ligase buffer	0.75
100X BSA	0.08
Ligase	0.50
BpiI	0.50
Level 2 Destination Vector (pAGM4723)	0.50
Level 1 Hygromycin Resistance (EC15030)	0.50
Level 1 SBPase (*A. thaliana* AT3G55800; EC23175)	0.50
End Linker (EC41766)	0.50

And added accordingly:

Reagent	**Volume (µL)**

Level 1 Constitutive AGPase (*A. thaliana* APL1 AT5G19220; EC23013)	0.50
Level 1 Green Tissue Specific AGPase (*A. thaliana* APL1 AT5G19220; EC23013)	0.50

Table 2.4: PCR recipe for Golden Gate cloning.

The final constructs consisted of the following:

1. The pL2V-pAGM4723 vector containing the NPTII gene for bacterial kanamycin resistance.
2. At position 1, the plant hygromycin resistance level 1 module EC15030. This module included a 35s promoter; a hygromycin resistance coding sequence derived from BRACT vectors (https://www.jic.ac.uk/research-impact/technology-research-platforms/crop-transformation/) and mutated so as not to contain BsaI or BpiI restriction sites; and a NOS terminator.
3. At position 2, the SBPase level 1 module EC23175. This modules included a 35s promoter; the *A. thaliana* SBPase coding sequence; and a NOS terminator.
4. At position 3, the AGPase level 1 module. For constitutive expression, this was module EC23013 and included a 35s promoter; the *A. thaliana* AGPase large subunit coding sequence (APL1); and a NOS terminator. For the tissue specific expression, this was module EC23295 and included a RuBisCO small subunit RbcS promoter; the *A. thaliana* AGPase large subunit coding sequence (APL1); and a NOS terminator.
5. An ELE end linker EC41766.

The plasmid with constitutive AGPase expression was named RE2-083 and the plasmid with tissue specific AGPase expression was named RE2-084. Full plasmid maps can be found in **Supplemental Figures 1 – 5**.

Successful cloning was confirmed by orange-white selection of transformed *E. coli* (see above protocol for transformation process). The pAGM4723 Level 2 vector contains the canthaxanthin operon which is removed during the cloning process. Synthesis of canthaxanthin causes the colony to develop an orange pigment. Therefore, only white colonies were selected as this indicated successful cloning. Successful cloning was further confirmed gel electrophoresis using an agarose gel (0.75 %, Fisher Bioreagents) made with TAE buffer (50 x dilution, Fisher Bioreagents) stained with GelGreen (10 µL L^{-1}, Biotium)

2.3.6 Sequencing

Plasmids were extracted and purified from transformed *E. coli* as directed by NucleoSpin Plasmid Easy Pure Kit (Macherey-Nagel). Plasmid minipreps were prepared as directed by Eurofins Genomics LIGHTRUN Sequencing Sample Requirements. Samples were shipped to and sequenced by Eurofins Genomics.

2.4 Transformation of Strawberry

2.4.1 Chemical transformation of Agrobacterium tumefaciens

Stock of *A. tumefaciens* (strain EHA105) stored in glycerol (25 %, Fisher Bioreagents) was thawed on ice. Plasmid of interest (1 µL, 100 ng mL^{-1}) was added to stock and rested on ice for 5 minutes. Mix was frozen in liquid nitrogen for 5 minutes and thawed in a water bath at 37 °C for 5 minutes. Mix was added to low salt LB broth (1 mL, Melford) and shaken at 28 °C for 2 hours. Bacteria were pelleted by centrifugation at 10,000 g for 2 minutes and re-suspended in low salt LB broth (100 µL, Melford). Bacterial suspension was plated on low salt agar plates with kanamycin (50 µg mL^{-1}, Duchefa) and incubated at 28 °C for 48 hours. Plates exhibiting successful colony growth were sealed with Parafilm and stored at 4 °C.

2.4.2 Strawberry micropropagation and growth conditions

Stocks of cultivated strawberry (*Fragaria x ananassa Duch.* cv. Calypso, EMR-773, EMR-2434) were donated by NIAB EMR. Stocks maintained on semi-solid Shoot Propagation Medium (SPM) and subbed every 4 weeks. Stocks bulked by 4 week growth on Shoot Multiplication Medium (SMM). Stocks grown in growth chamber at 20 °C under ~68 µmol m^{-2} s^{-1} light intensity with a 16/8 photoperiod. All other conditions were ambient. See **Tables 2.5 – 2.7** for media recipes.

Table 2.5 – SPM Media Recipe and FRAG-R Media Recipe

Honey jars ~50ml/jar	**Stock**	**mL L^{-1} OR g L^{-1}**
MS with vitamins	Powder	4.4 g
Sucrose	Household sugar	30 g
BAP (6-benzylaminopurine)	1 mg/ml	0.1 mL
IBA (Indole-3-butyric acid)	1 mg/ml	0.1 mL
pH	N/A	pH 5.8
Agar Daishin	Powder	9 g

FRAG-R media recipe: SPM recipe with half concentrations of MS with vits and sucrose

Table 2.6 – SMM Media Recipe

Honey jars ~50ml/jar	**Stock**	**mL L^{-1} OR g L^{-1}**
MS with vitamins	Powder	4.4 g
Sucrose	Household sugar	30 g
BAP (6-benzylaminopurine)	1 mg/ml	0.5 mL
pH	N/A	pH 5.8
Agar Daishin	Powder	9 g

Table 2.7 – SRM Media Recipe

Plates ~25ml/plate	**Stock**	**mL L^{-1} OR g L^{-1}**
MS with vitamins	Powder	4.4 g
NAA (1-naphthaleneacetic acid)	1 mg mL^{-1}	0.2 mL
TDZ (Thidiazuron)	1 mg mL^{-1}	1 mL

Make to 90 % final volume with dH_2O

pH 5.8	N/A	pH 5.8
Agargel	Powder	5 g

After autoclaving add:

Filter-sterilised glucose solution	30 g 100 mL^{-1}	100 mL

For plates with selectable markers add:

TCA (Ticarcillin/clavulanic acid)	400 mg mL^{-1}	1 mL
Kanamycin	50 mg mL^{-1}	0.5 mL
Hygromycin	25 mg mL^{-1}	0.25 mL
Glufosinate	5 mg mL^{-1}	0.05 mL

2.4.3 Transformation of strawberry explants

Protocol adapted from Schaart, 2014. Using a sterile pipette tip, individual transformed colonies of *A. tumefaciens* were collected and added to low salt LB broth (5 mL, Melford) with kanamycin (50 µg mL^{-1}, Duchefa) and rifampicin (25 µg mL^{-1}, Duchefa). Samples were shaken at 28 °C overnight. Starter culture (1 mL) was added to low salt LB broth (20 mL, Melford) with kanamycin (50 µg mL^{-1}, Duchefa) and rifampicin (25 µg mL^{-1}, Duchefa) and shaken at 28 °C overnight. Cultures were pelleted at 2000 xg for 10 minutes. A suspension medium of filter-sterilised MS with vitamins (4.4 g L^{-1}, Duchefa) supplemented with glucose (30 g L^{-1}, Fisher Bioreagents) and acetosyringone (100 µM, Duchefa) and pH adjusted to 5.2 was made. Bacterial pellets were re-suspended in sufficient suspension medium to give OD 600 nm 0.2 – 0.3. Young expanding leaves of 4 week old strawberry plants were separated into leaflets and scored to produce ~2 mm thick strips along the leaf edge. Scored leaflet explants were submerged in the inoculum suspension for 10 – 15 minutes. Treated explants were blotted on sterile filter paper and placed, abaxial face up, on a sterile filter paper on Shoot Regeneration Medium (SRM, see recipe in Appendix 1). Explants were stored in the dark for 4 days. Explants were then treated with a solution of filter-sterilised ticarcillin disodium/clavulanate potassium (TCA, 400 mg L^{-1}, Duchefa), blotted on sterile filter paper and transferred to SRM plates containing hygromycin (25 µg mL^{-1}, Duchefa) or glufosinate ammonium (5 µg mL^{-1}, Sigma) (depending on the selectable marker present in the plasmid backbone) and TCA (400 µg mL^{-1}, Duchefa). Plates were sealed with 3M micropore tape and placed in a growth chamber at 20 °C under ~68 µmol m^{-2} s^{-1} light intensity with a 16/8 photoperiod. All other conditions were ambient.

2.4.4 Regeneration of strawberry explants

Transformed explants were transferred to fresh SRM with selectable markers every 4 weeks, abaxial face up. Control explants were cultured without selectable markers. Growing calli were divided as appropriate to maintain contact with the culture medium. Plants with regenerated leaf tissue and visible crown development were excised and transferred to rooting medium (FRAG-R) with selectable markers for transformed plants. Plants were left on FRAG-R until strong root development was visible or until media needed changed (every four – six weeks). Once fully regenerated, transformed plants were moved to SMM without selectable markers to bulk up crown numbers before being transferred to SPM for regular propagation.

2.5 Analysis of Transgenic Strawberry Plants

2.5.1 Weaning of micropropagated plants onto soil

In preparation for weaning, plants were propagated onto FRAG-R to encourage root development. Plants were left to grow until several roots (approximately > five) were visible in the media up to and beyond five centimetres in length. This usually took four – eight weeks. Once roots had sufficiently developed, plants were planted into 24 cell trays (Nutley's, 36 cm x 24 cm x 5cm) and filled with well-watered, medium grain vermiculite (Sinclair Pro). A hole was pressed into the vermiculite using a spatula and the plant roots pressed into the base of the tray using tweezers. The vermiculite was then pressed down around the roots, ensuring that the crown was not buried to prevent rot. Trays were placed into propagators with vents (Stewart, 52 cm x 42.5 cm x 28 cm) and the vents closed. After one week, vents were opened half way. After two weeks, vents were fully opened. After three weeks, the lid was removed entirely. Plants remained on vermiculite for two further weeks. Plants were transplanted into well-watered soil (Sinclair) by pressing a hole into a soil filled pot (Soparo, 1 L), pushing roots into the base of the hole and then packing the soil around the plant ensuring the crown was not buried to prevent rot. Plants were moved to larger pots (Soparo, 2 L) after four weeks of growth using the same method to avoid becoming pot bound.

2.5.2 Plant growth conditions

Plants were grown in a growth chamber with overhead white fluorescent lighting emitting at 200 µmol m^{-2} s^{-1}, a 16/8 hour photoperiod (8:00 AM – 12:00 AM) and at 25 °C. Plants were watered via mattress watering while planted in 1 L pots to encourage root development and via overhead watering while planted in 2 L pots. Runners were cut every four weeks to prevent overgrowth. When new plants were needed for analysis, runners with visible shoot

development were planted onto well-watered, soil filled pots (Soparo, 1 L) and pinned down using pegs. After four weeks of growth, explants were severed from the parent plant.

2.5.3 Harvesting of transgenic leaf material

Young, fully expanded, non-senescent leaves were selected for harvesting. Leaves were harvested by cutting into 3 smaller segments, during which the midrib was removed, placed in 2 mL collection tubes and immersed immediately in liquid nitrogen to limit degradation of the RNA. Leaf samples were then stored at -80 °C until RNA extraction.

2.5.4 RNA extraction and analysis

RNA was extracted using the NucleoSpin RNA Plant and Fungi Kit (Macherey-Nagel) according to the kit instructions. Concentration and quality of extracted RNA was determined using a Nanodrop 1000 spectrophotometer (Thermo scientific) to confirm sufficient purity for cDNA construction.

2.5.5 Reverse Transcription

cDNA was synthesised using the High-Capacity cDNA RT kit (Applied Biosystems) according to the kit instructions. In each instance, 10 µL RNA was processed. Resulting cDNA was analysed using the Nanodrop 1000 spectrophotometer (Thermo scientific) and diluted to 5 ng mL^{-1} using dH_2O.

2.5.6 Quantitative PCR

Primers for qPCR were designed using NCBI Primer-Blast for *A. thaliana* SBPase and AGPase and *Fragaria x ananassa. (Duch)*. *A. thaliana* sequence was derived from plasmid sequences. *F x ananassa* sequences were derived from the *Fragaria x ananassa* Camarosa Genome v1.0.a2 (Liu *et al.*, 2021). *F x ananassa* specific housekeeping gene primers were taken from Lin *et al.*, 2021 and Clancy *et al.*, 2013. Primer sequences were as follows:

Species	**Direction**	**Gene**	**Sequence (5' – 3')**
Arabidopsis thaliana	Forward	SBPase	TGCGATACACCGGAGGAATG
	Reverse	SBPase	GGTCTTGTCAAGCACGGACT
	Forward	AGPase	GTTCCTATCGGGGGAGCCTA
	Reverse	AGPase	TTCTCCTGGCGTTTGAGTGG
Fragaria x ananassa	Forward	SBPase	GCACCCACGAGTTCCTTCTT
	Reverse	SBPase	GGTTAACGTCGGGAACCAGT
	Forward	AGPase	AAGCGAATAGAGGGCTTTCCT
	Reverse	AGPase	CAACACACTTCTGCTCGCGT
	Forward	26srRNA	ACCGTTGATTCGCACAATTGGTCATCG
	Reverse	26srRNA	TACTGCGGGTCGGCAAACGGGCG
	Forward	GAPDH1	CATTGAGAGCAGGCAGAACCT
	Reverse	GAPDH1	CCTCATTCAACATCATTCCTAGCA
	Forward	ENP1	GCCACGTCTCTTTGACATTGACT

	Reverse	ENP1	TTCCGAATGGGCTTTCCA

Table 2.8: Primer sequences for qPCR.

Primers were diluted to 10 µmol. A master mix using the SYBR Green qPCR Master Mix Kit (Applied Biosystems) was made as follows for a total reaction volume of 10 µL:

Reagent	**Volume (µL)**
SYBR Green qPCR Master Mix	5
Forward Primer	1
Reverse Primer	1
cDNA	2
dH_2O	1

Table 2.9: PCR recipe for qPCR.

Samples were pipetted into a white rectangular 96-well plate (Bio-Rad) covered with transparent plastic (Bio-Rad). Plates were loaded into the CFX Opus 96 Real-Time PCR System (Bio-Rad) for analysis. The qPCR programme was as follows:

1. 95 °C for 10 minutes;
2. 40 cycles of 95 °C for 15 s followed by 60 °C for 60 s;
3. A temperature ramp from 65 °C – 95 °C at a rate of 0.5 °C s^{-1}.

Fluorescence data was extracted to Microsoft Excel for processing.

2.5.7 Chlorophyll fluorescence analysis

Four week old plants propagated on runners (see 2.5.2) were selected for analysis, taken to the University of Essex and placed in a growth room under the same conditions as in 2.5.2. Before analysis, plants were kept in darkness for 30 minutes for dark adaption. Images were taken using the CF Imager (Technologica). Saturating pulse was set at 6354 µmol m^{-2} s^{-1} for 800 ms PPFD. Following dark adaption, two plants were placed in the imager such that one whole tripartite leaf was visible on each plant. Selected tripartite leaves were measured to approximately 10 cm in diameter and visually observed to ensure no damage and similar leaf age. Young, fully expanded, non-senescent leaves were selected for analysis. The camera aperture was adjusted to ensure visual clarity of the leaves and background strays removed from the image. Once completed, set up was left for five minutes to ensure stability of the recorded fluorescence signal. Plants were then treated to the following regimen:

1. Single saturating pulse for measurement of F_0, F_m and F_v.
2. Actinic light applied at 400 µmol m^{-2} s^{-1} for 10 minutes with saturating light applied at one minute intervals to measure changes in F_m' and F_q'.
3. Actinic light applied at 800 µmol m^{-2} s^{-1} for 10 minutes with saturating light applied at one minute intervals to measure changes in F_m' and F_q'.

F_0' and F_v' were calculated by the imaging software.

2.6 Statistical Analysis

Statistical analysis was carried out using Microsoft Excel and R (https://www.r-project.org/). Welch's t-tests, paired t-tests and binomial tests were applied where appropriate to compare statistically significant differences between data sets. Error bars throughout were calculated to show standard error of the mean unless otherwise specified. Letters above text indicate significantly different values to the 5 % significance level. Asterisks indicate significance as defined in individual figures.

Chapter 3 – Exploring variation in photosynthetic carbon assimilation, intrinsic water use efficiency and fruit yield across the east-west axis of polytunnel grown cultivated strawberry

3.1 Introduction

Cultivated strawberry (*Fragaria x ananassa Duch.*) is a nutritious fruiting crop with high value to the UK fruit industry. Strawberry production in the UK was worth an estimated £352.4 million in 2021, representing an estimated 24.5 % of the total value of UK fruit production (DEFRA, 2022). Strawberries themselves contain high concentrations of vitamin C, minerals and antioxidant phenolic compounds that have been demonstrated to confer health benefits (Giampieri *et al.*, 2014), such as significantly reduced blood cholesterol in human subjects who consumed 500 g strawberries daily for one month (Alvarez-Suarez *et al.*, 2014). Work carried out *in vitro* on cell cultures and *in vivo* on rats has demonstrated the potential benefit of antioxidant phenolic compounds in preventing incidence of cancer (Wang *et al.*, 2013) and improving mitochondrial respiratory function (Giampieri *et al.*, 2013). Protecting current yields and increasing future yield of UK strawberry production could therefore have a marked effect for improving both medical and economic health.

Strawberry production in the UK usually occurs on raised beds under polytunnels. It is estimated that greater than 50 % of strawberry production occurs in substrate rather than soil, usually the substrate coir derived from coconut husks (Robinson Boyer *et al.*, 2016). An estimated 114.6 thousand tonnes of strawberry fruit was produced in 2021 over 4931 hectares of land (DEFRA, 2022). This production is highly influenced by microclimate; this in turn is highly

influenced by growth beneath a polytunnel. Previous work has shown that growth beneath a covered polytunnel increased fruit yield by 40 % across three years of study in five everbearing cultivars compared to identical table-top production beneath an uncovered polytunnel (Condori *et al.*, 2017). Similarly, strawberry plants grown in field conditions versus those grown on raised beds beneath a polytunnel had reduced yield, longer time to flowering (five weeks) and a reduced soluble solids content (Kadir, Carey, *et al.*, 2006). This highlights the importance of polytunnels for increasing strawberry production. There is significant variation across the polytunnel climate however, resulting in a series of microclimates across the tunnels length and width. For example, pollinators have been shown to preferentially make more visits to flowers at the ends of polytunnels, reducing pollination rates in the polytunnel centre and leading to reductions in fruit size, mass and crumbliness in raspberry (a mark of fruit quality) (Hall *et al.*, 2020). Furthermore, presence of weeds in polytunnels may reduce visits to strawberry flowers due to pollinator preference for weed flowers (Howard *et al.*, 2021). These studies demonstrate that, while growth beneath a polytunnel already leads to large gains in strawberry productivity, much remains to be optimised for maximal yield.

As previously discussed, photosynthetic efficiency represents an as yet un-optimised target for improving yield (Long *et al.*, 2006). Increased availability of photosynthetically active radiation (PAR) has been linked to increased yield in strawberry (Palencia *et al.*, 2013) and increased rate of fruit ripening (Twitchen *et al.*, 2021); understanding this relationship could improve reliability of forecasting fruit production. In light of this, work has been carried out to understand how fruit photosynthesis is impacted by polytunnel and glasshouse shading, although none of this has been carried out in strawberry. Previous work has identified increased annual total PAR for greenhouses built along a north-south axis, although more PAR is available in winter for greenhouses built along the east-west axis at high latitudes (Robson *et al.*, 2022). PAR may also be affected by polytunnel material and structure, the type of crop grown and planting regimens (Robson *et al.*, 2022). Strawberry in particular may well be affected by canopy

shading of lower leaves reducing photosynthetic efficiency at different times of day (Li and Yang, 2015). This effect has been seen in tomato, where the use of films to increase diffusion of light allowed for greater penetration of the lower canopy and resulted in 5.3 % increase in carbon uptake and a 4.6 % increase in light use efficiency (Shin *et al.*, 2021).

This chapter aims to understand how polytunnel microclimate affects strawberry photosynthesis and yield. Historical data collected by the WET Centre (not shown) has shown an elevated fruit yield of ~ 10 % in the westernmost of the two central rows in a six row tunnel (namely Row 4, see **Fig. 2.2**). It has been hypothesised that this could be correlated with increased photosynthesis due to this row receiving greater PAR, leading to greater carbon assimilation and synthesis of harvestable biomass. This is due to the wide alley down the centre of the polytunnel reducing shading on plants grown in Row 4 during the morning, when the sun is in the east. Understanding this could inform better agronomic practice for improved strawberry yields, generate greater understanding of the relationship between PAR and yield beneath a polytunnel and highlight photosynthetic factors that may limit strawberry productivity. Measurements were taken during the first and second fruiting flushes, termed the "early season" and the "mid-season" from here on in.

The hypotheses to be considered in this chapter are as follows:

- Row 4 receives the greatest PAR.
- Row 4 displays the greatest capacity for photosynthetic carbon assimilation.
- Row 4 has the greatest total fruit yield.

3.2 Materials and Methods

Materials and methods were carried out as described in Chapter 2, section 2.2.

3.3 Results and Discussion

3.3.1 Effect of polytunnel row position on photosynthetic carbon assimilation

3.3.1.1 Row 4 receives significantly greater PAR earlier in the year than other rows and inner and outer rows segregate

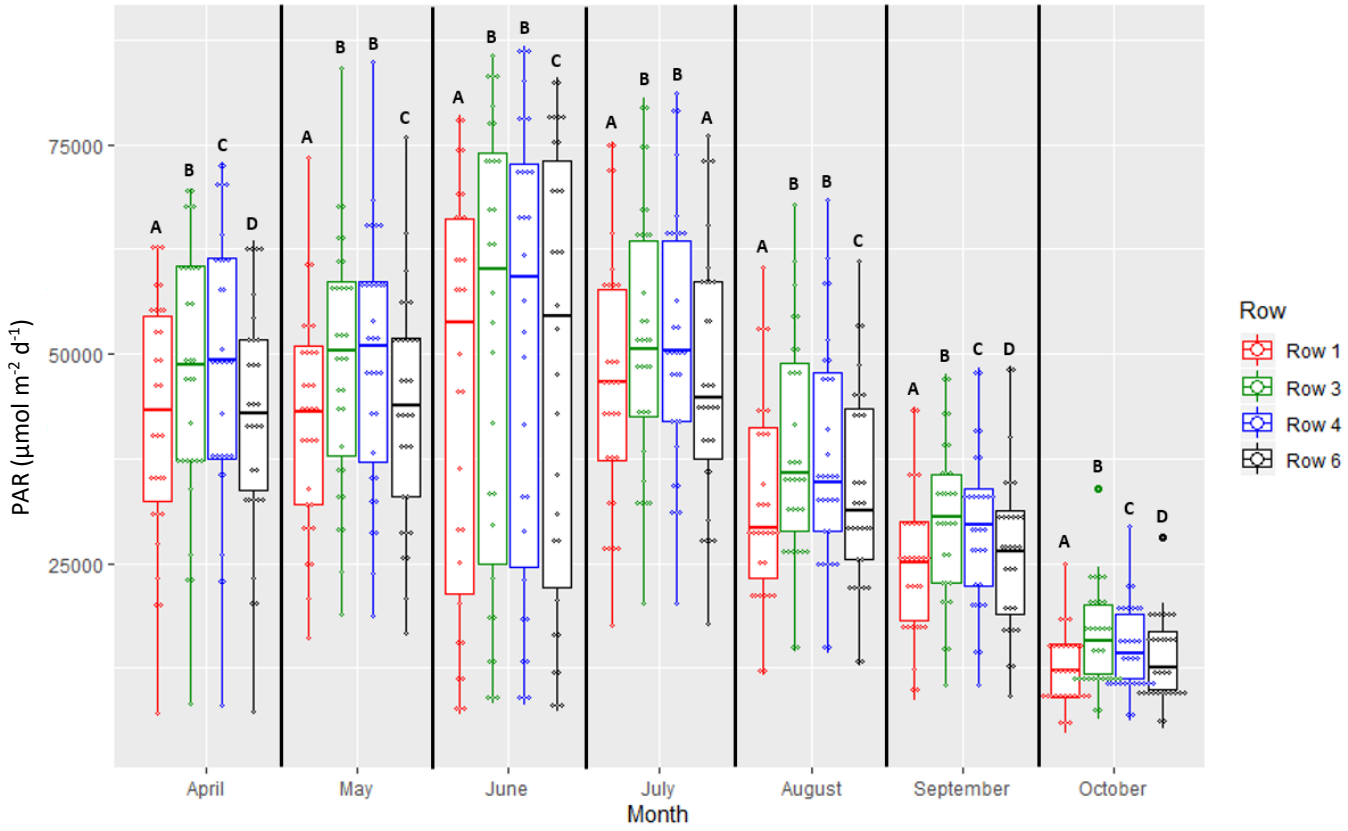

Fig. 3.1: Daily PAR received by row each month April – October 2021. Different letters show significantly different daily PAR analysed on a month-by-month basis (paired T-test, n = 30 – 31, $p < 0.05$).

Throughout the growing season, PAR by row was measured (**Fig. 3.1**). Daily PAR was recorded and statistically compared on a month-by-month basis using a paired T-test to account for daily variation in weather that affected all rows. This revealed some interesting insights. Row 4 received significantly greater PAR than all other rows in April and Row 3 significantly greater PAR than all other rows in September and October. Internal rows always received significantly more PAR than external rows and Row 1 always received significantly less light than all other rows (with the exception of July, where it received the same quantity of PAR as Row 6).

The greater light received by Row 4 in April is of particular import for two reasons. Firstly, greater available PAR can enable greater photosynthesis at this time, which has been associated with an increase in dry matter production (Sivakumar and Virmani, 1984). Since this is occurring early in the growing season, this could result in Row 4 plants developing larger canopies for greater light interception later in the season, allowing them to perform more total photosynthesis than other rows (given photosynthetic efficiency is similar). Secondly, greater PAR drives greater floral initiation (Wang *et al.*, 2020) and has been associated with a greater number of fruit bearing trusses in everbearing strawberry (Maas and Cathey, 1987). The higher quantity of PAR in Row 4 in April (and, more generally, in the central rows across the season) may contribute to a greater number of available flowers that could be pollinated for fruit production. From this, it is expected to see a greater number of fruits produced by the central rows than the external rows with Row 4 having the greater number of fruits overall. It should also be noted that, following this theory, Row 1 will produce the least number of fruits (due to the low PAR it receives).

3.3.1.2 Strawberry photosynthetic rate varies with time of day and season

Point measurements were taken at 6 time points over the course of the day at saturating light to determine daily fluctuations in strawberry leaf photosynthesis (see **Fig. 3.2**). In both the early and the mid-season, photosynthetic carbon assimilation rate was greatest by 11:00 AM and the reduced through the afternoon and evening. During the early season (**Fig. 3.2.A**), photosynthetic rate increased more rapidly, reaching its greatest rate by 8:30 AM compared to the significantly lower rate (T-Test, $p < 0.05$) at this time during the mid-season (**Fig. 3.2.C**). Despite this, the total quantity of daily assimilated carbon is not significantly different between the early and mid-season (**Fig. 3.2.B/D**, T-Test, $p < 0.05$). There was also no significant difference in total carbon assimilated observed between rows.

The diurnal pattern observed here is expected and typical. Changes in photosynthesis across a day are theorised to balance assimilation with water loss (Buckley, 2017) and are driven by stomatal activity and RuBisCO activation (Matthews *et al.*, 2017). Greater assimilation in the morning is stimulated by fully open stomata and a lack of end product inhibition, allowing for leaves to make full use of available light. Following midday inhibition and the accumulation of end products, negative feedback occurs to reduce activity of the CBC. This also leads to a reduction in stomatal aperture, since maintaining a high stomatal aperture results in greater water loss than is worth the low returns in fixed carbon. This results in the gradual decline across the afternoon as observed here.

However, previous work examining the pattern of diurnal photosynthesis for strawberry grown in a controlled environment is surprisingly contradictory to this concept. One study found that net photosynthetic rate peaked around 9 AM then declined steadily throughout the day (Garcia and Kubota, 2017), whereas another identified peak photosynthesis to occur around midday (Wu *et al.*, 2012), although photosynthesis was almost at its peak by 9 AM. This highlights the inconsistency of controlled environment studies on photosynthetic physiology

identified previously (see Chapter 1, section 1.3.1). The results presented here suggest a photosynthetic peak around 11 AM, closer to the latter study, and underlines the importance of field work for greater understanding of how plant physiology responds in a setting closer to commercial agronomy. The diurnal pattern reported here has also been reported to occur in other crops such as maize (Hirasawa and Hsiao, 1999; Kalt-Torres *et al.*, 1987) and grape (Downton *et al.*, 1987). Interestingly in grape, vines with no fruit fixed 22 % less carbon. This could be due to reduced sink-strength from fewer carbon sinks (i.e. fruits) available. Reduced sink-strength in strawberry has previously been shown to reduce fruit yield and is therefore a similarly limiting factor (Sønsteby *et al.*, 2021), but the effect this has on photosynthesis of fruitless plants has so far given inconclusive results (Ferree and Stang, 1988).

While there is no significant difference between photosynthetic carbon assimilation of different polytunnel rows through this measurement, it is notable that the eastern rows (1 and 3) tended to perform better than other rows when the sun was in the east and vice versa for the western rows (4 and 6) which could warrant further study. The overall lack of difference between rows across the day may be explained by significant leaf-to-leaf variation as a result of unequal development between plants; growth of leaves shaded by the upper canopy can effect large differences in photosynthetic capacity (Lawson *et al.*, 2012). While efforts were taken to reduce this variation through selecting leaves of similar size, age and canopy position for analysis, a dynamic field environment with great variation in plant development has a greater likelihood of producing leaves with a large leaf-to-leaf variation in photosynthesis.

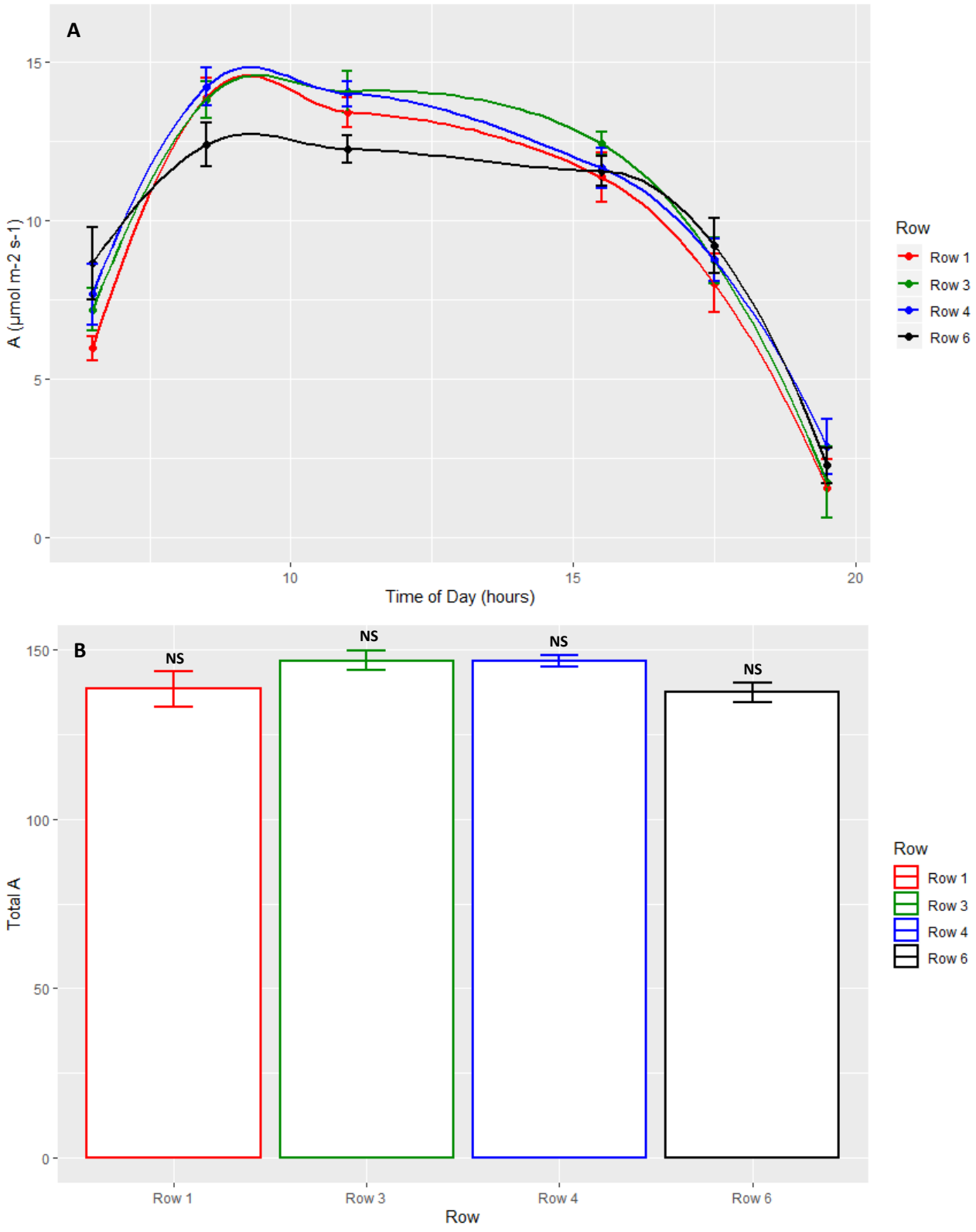

Fig. 3.2 A – B: Point data showing variation in strawberry leaf carbon assimilation across a day in four different polytunnel rows. **A:** Early season (beginning of fruiting, early/mid-June). **B:** Daily average total assimilated carbon as defined as area under the curve of 3.2.A. n = 16 – 24, error bars show standard error.

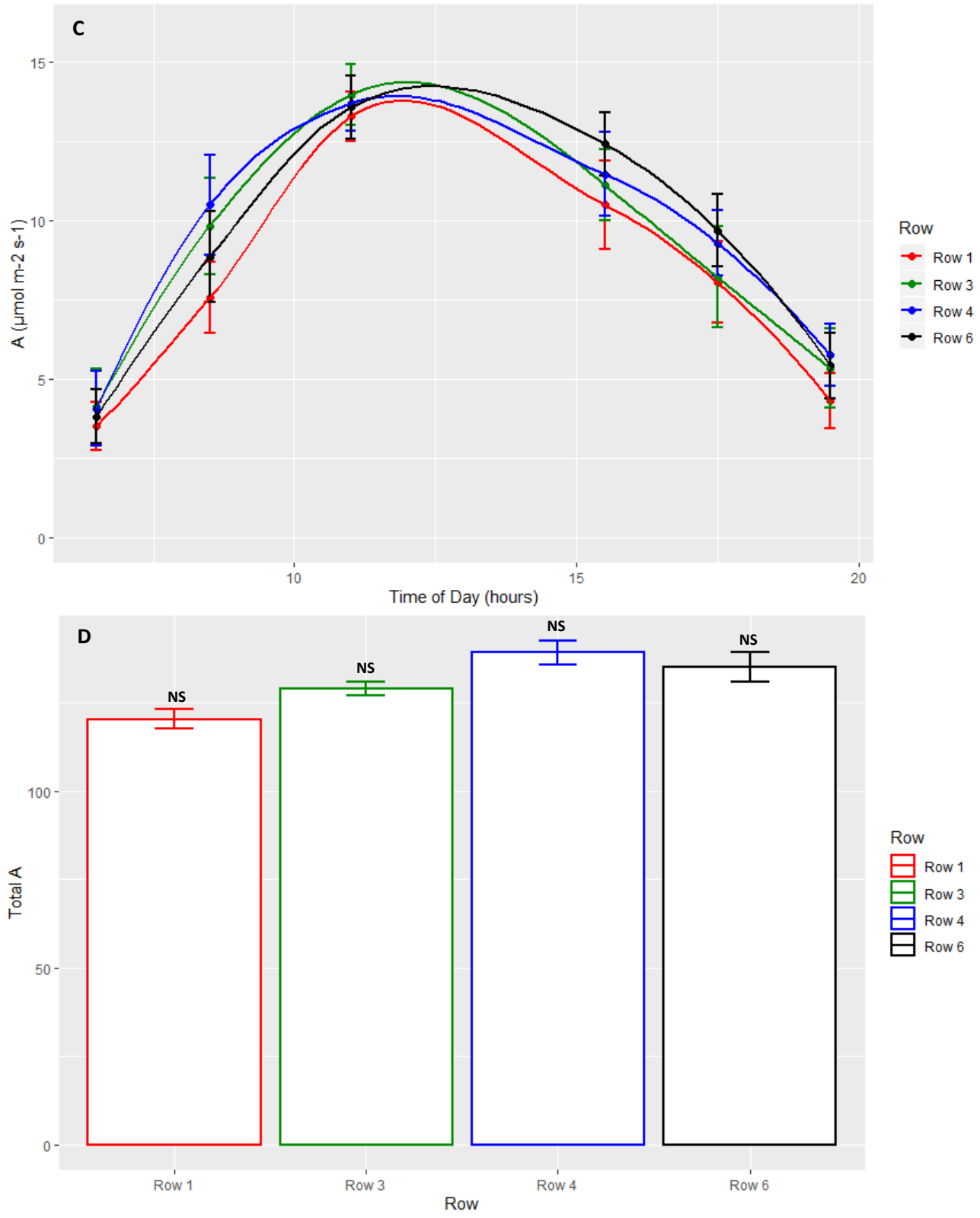

Fig. 3.2 C – D: Point data showing variation in strawberry leaf carbon assimilation across a day in four different polytunnel rows. **C:** Mid-season (middle of fruiting period, early August). **D:** Daily average total assimilated carbon as defined as area under the curve of 3.2.C. n = 16 – 24, error bars show standard error.

3.3.1.3 Photosynthetic efficiency of strawberry leaves differs between rows and Row 4 has the greatest carbon assimilation in the early season

To investigate maximal leaf photosynthetic capacity, measurements were taken using a light response curve. The advantage of this approach is that the leaf is able to acclimate to saturating light before measurements are taken at lower light levels. As this means enough photosynthetic machinery will be activated to make full use of the available light at each saturation point, this gives an indication of the best possible leaf response at different light levels. This is important to understand in a field environment, where PAR fluctuates both across a day and across a growing season, and especially in a polytunnel where PAR can be reduced by up to 34 % (Condori *et al.*, 2017). To this end, it is also important to understand how field grown crops respond at different points of the growing season, hence the use of two time point measurements in this experiment. Saturating light was set at 1500 µmol m^{-2} s^{-1}, since saturating light in strawberry has previously been identified to occur at 1400 µmol m^{-2} s^{-1} (Carlen *et al.*, 2009).

The light saturated rate of photosynthetic carbon assimilation (A_{max}) is similar to previously reported measurements in strawberry (~15 – 20 µmol m^{-2} s^{-1}, Fig. 3.3.A) (Ferree and Stang, 1988). In the early season, Row 4 was identified as having total 9 % - 20 % greater carbon assimilation than all other rows in the morning (**Fig. 3.3.B**) but this difference was not significant – further repeats of this experiment are necessary to confirm or deny if this increase is truly present It is notable that this increase in photosynthesis occurs during the morning since a previous study highlighted that, due to increased negative feedback on photosynthesis, net photosynthetic rate during the morning is a major limiting factor on strawberry photosynthesis (Garcia and Kubota, 2017). Since increased photosynthetic rates have previously been linked to increased yield in strawberry (Choi *et al.*, 2016), this may go some way to explaining historical yield patterns.

At light saturation of ~1300 µmol m^{-2} s^{-1} and greater, Row 4 displayed slightly significantly greater capacity for photosynthetic carbon assimilation than Rows 3 and 6 (T-Test, $p < 0.1$) (**Fig. 3.3.A**), although the total quantity of carbon assimilated wasn't significant across all light levels taken together. This demonstrates that Row 4 is better equipped to make use of high levels of PAR, since elevated PAR is correlated with increased photosynthesis (Decoteau, 1998). Furthermore, this relationship has also been demonstrated specifically in strawberry. Increased light intensities have been found to increase net photosynthetic rates of strawberry leaves grown under polytunnels (Oda, 1997) and fruit yield of strawberry plants grown under supplemental lighting in controlled conditions (Hidaka *et al.*, 2013; Yang *et al.*, 2022). As Row 4 receives greater PAR due to its row position, this may further explain how Row 4 is able to achieve greater yields than other polytunnel rows. A different explanation for increased Row 4 yields may relate to improved environmental tolerances. A recent study found that supplemental lighting at 200 µmol m^{-2} s^{-1} at a variety of visible light wavelengths enhanced carbon assimilation relative to control plants under conditions of salt and alkali stress (Shamsabad *et al.*, 2022). For plants experiencing abiotic stresses in the field, the elevated PAR and carbon assimilation experienced by Row 4 plants may aid in reducing yield losses.

In the afternoon however, a different picture emerges (**Fig. 3.3.C**). While Row 4 still exhibits a high capacity to make use of available PAR in the afternoon, Row 6 also displays high capacity for photosynthetic carbon assimilation at high levels of PAR and only Row 1 is significantly different to the other three rows. Additionally, total assimilated carbon (**Fig. 3.3.B/D**) is significantly reduced in all rows in the afternoon with the exception of Row 3 (T-Test, $p < 0.05$). This may be triggered by the accumulation of photosynthetic end products, such as starch and sucrose, that inhibit photosynthetic enzyme activity and by the closing of stomata to conserve water when these enzymes are inhibited, reducing available atmospheric carbon for fixation (Geiger and Servaites, 1994).The complete decline in photosynthetic capacity of Row 1 is unusual. As it stands, there is little reasonable biological explanation as to why the assimilation

in Row 1 is so poor in this set of measurements, especially given the randomised block design incorporated into the methodology. It may relate to some further element of shading, reducing availability of PAR, end product limitation, or a different limiting factor, such as temperature. Despite this, Row 1 could make an interesting observation for understanding limitations to strawberry photosynthesis.

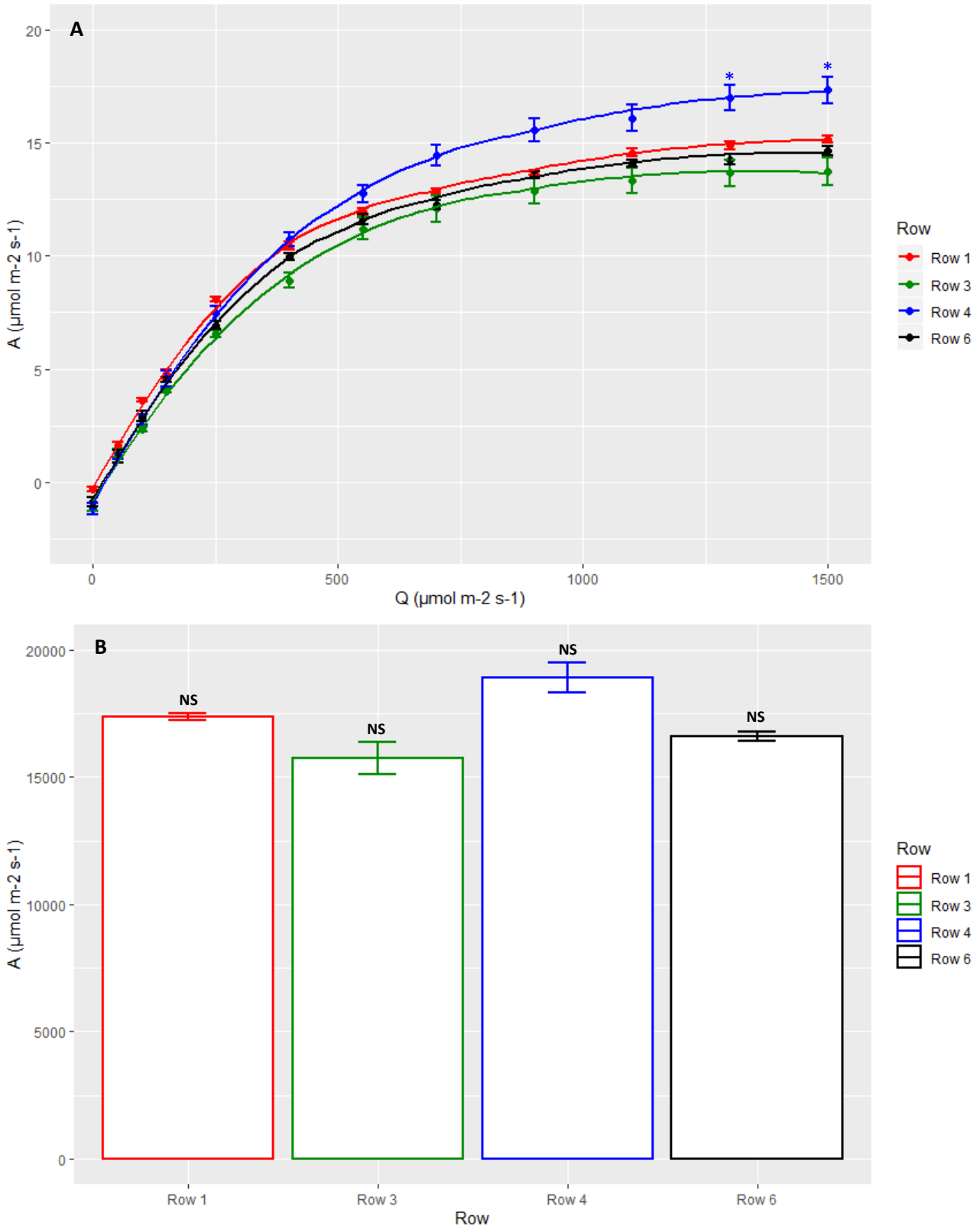

Fig. 3.3 A – B: Light response curves showing variation in strawberry leaf carbon assimilation in four different polytunnel rows in the early season (beginning of fruiting, early/mid-June). **A:** Morning measurements (7 AM – 11 AM). **B:** Integrated area under the curve of Fig 3.3.A. n = 4, error bars show standard error. * indicates intensities at which Row 4 is significantly different from other rows (T-Test, n = 4, $p < 0.1$).

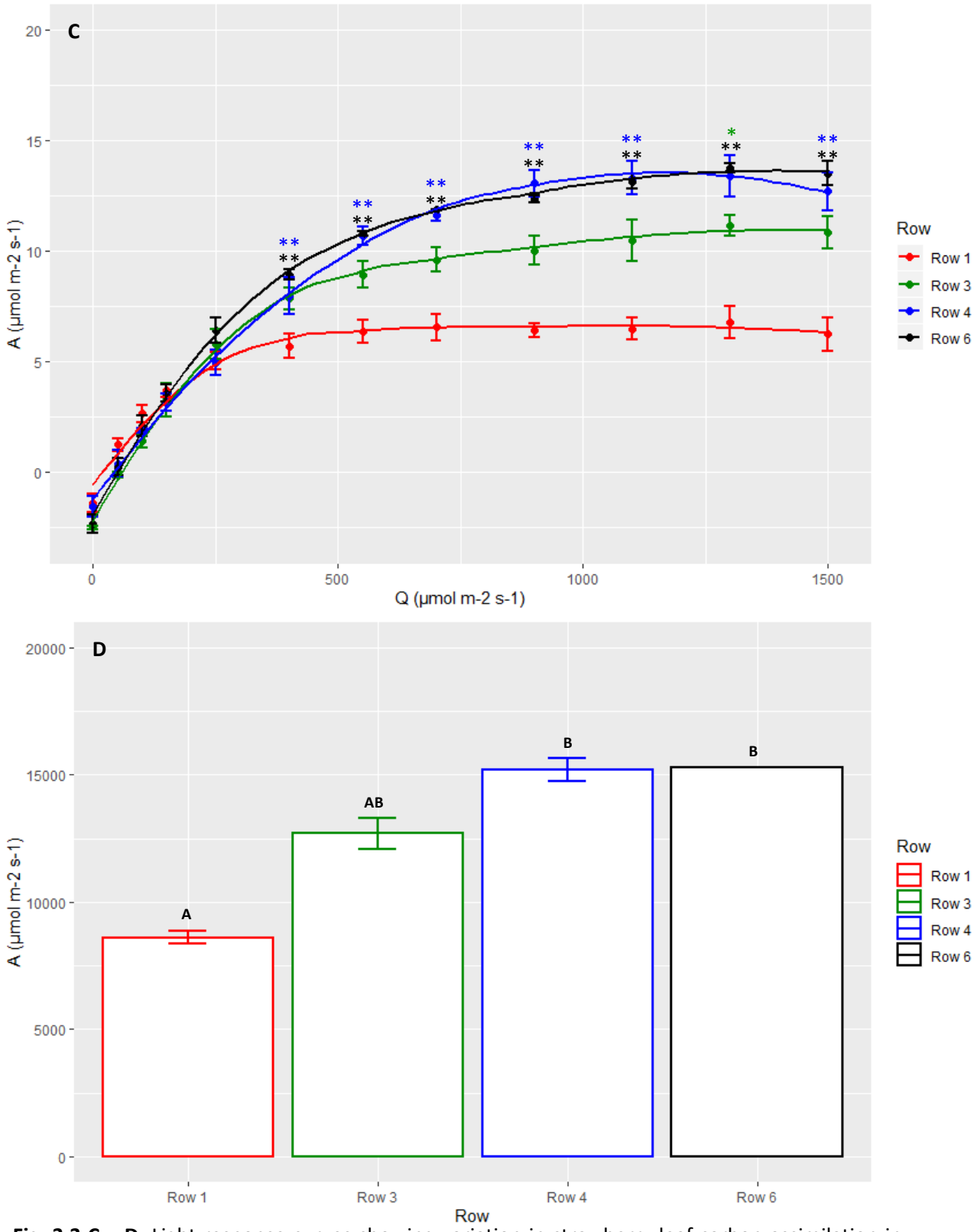

Fig. 3.3 C – D: Light response curves showing variation in strawberry leaf carbon assimilation in four different polytunnel rows in the early season (beginning of fruiting, early/mid-June). **C:** Afternoon measurements (3.30 PM – 7.30 PM). **D:** Integrated area under the curve of Fig 3.3.C. n = 4, error bars show standard error. * = significant at 10 % level, ** = significant at 5 % level, both relative to Row 1, colours match Row key.

3.3.1.4 Photosynthetic capacity of strawberry leaves is reduced in the mid-season and Row 4 no longer has the greatest rate of carbon assimilation

As previously stated, it is important to understand if the patterns previously explored are repeated at different time of the growing season. This is to enable a more tailored response to different conditions to maximise photosynthetic capacity.

It is apparent from this work that the patterns seen in the early season are not replicated later in the season. During the mid-season, in the morning Row 4 no longer has the greatest capacity for photosynthetic carbon assimilation (**Fig. 3.4.A – B**). It is notable that Row 3 has a similar degree of total carbon assimilation as Row 4, since the fruit yield of these two rows is also very similar by the end of the season and these rows receive similar PAR between May and August. It should also be noted that Row 1 exhibits a considerably lower assimilation rate that all other rows in saturating light.

During the afternoons of the mid-season (**Fig. 3.4.C – D**), Row 4 has the lowest assimilation of all rows at greater levels of PAR (~750 µmol m^{-2} s^{-1} and above), with this difference being significant at the highest light intensities. During the afternoon, photosynthetic rate decreases and the low carbon assimilation seen in Row 4 during this time may be due to this diurnal change rather than an overall reduced rate of photosynthetic carbon assimilation across the day; Row 4 still exhibits a high carbon assimilation rate in the morning and A_{max} is significantly greater in the morning than the afternoon (T-Test, $p < 0.05$).

Across the mid-season, A_{max} is generally lower than across the early season. This may be due to cooler and less favourable temperatures for photosynthesis, reduced day length decreasing total availability of PAR across a day or a combination of these factors. Leaf age may also have an impact here, however it has been demonstrated that photosynthesis in 30 day old fully expanded leaves is up to 24 % greater than new, fully expanded leaves (Carlen *et al.*, 2009). This may suggest photosynthetic rate should increase in the mid-season, when leaves have fully

developed and sink-strength is at its greatest. This trend is not seen here. This could be explained due to this study exploring the effects of leaf age on Junebearing varieties; a different effect may be present in everbearing varieties such as the cultivar Malling Centenary used in this experiment.

Since measurements were not taken over midday, any effect of midday inhibition could not be observed. Midday inhibition is a well-documented phenomenon where photosynthetic carbon assimilation rates dip in the middle of the day (Tenhunen *et al.*, 1987). It is possible that, given the high light intensities during the growing season, that the strawberries grown here may have experienced midday inhibition. Around midday, leaf temperatures and air-to-leaf water vapour pressure deficits are at their greatest, triggering stomatal closure and reducing photosynthetic carbon assimilation (Maai *et al.*, 2020). It is theorised that this can also increase susceptibility to photodamage (Maai *et al.*, 2020). For measurements taken in the afternoon then, if significant photodamage has occurred, this may explain reduced rates observed. Midday measurements may be valuable in exploring this concept further.

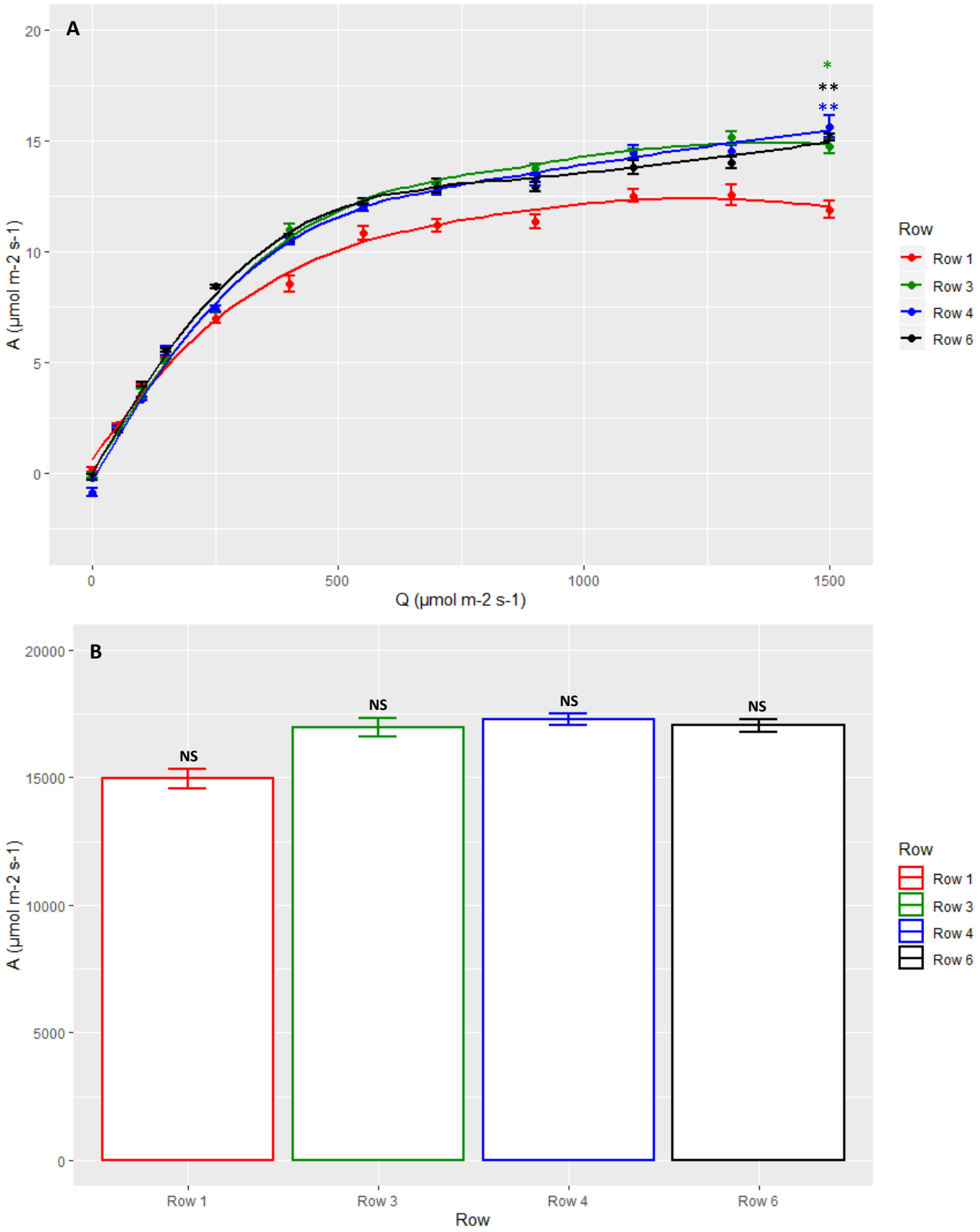

Fig. 3.4 A – B: Light response curves showing variation in strawberry leaf carbon assimilation in four different polytunnel rows in the mid-season (middle of fruiting period, early August). **A:** Morning measurements (7 AM – 11 AM). **B:** Integrated area under the curve of Fig 3.4.A. n = 6, error bars show standard error. * = significant at 10 % level, ** = significant at 5 % level, both relative to Row 1, colours match Row key.

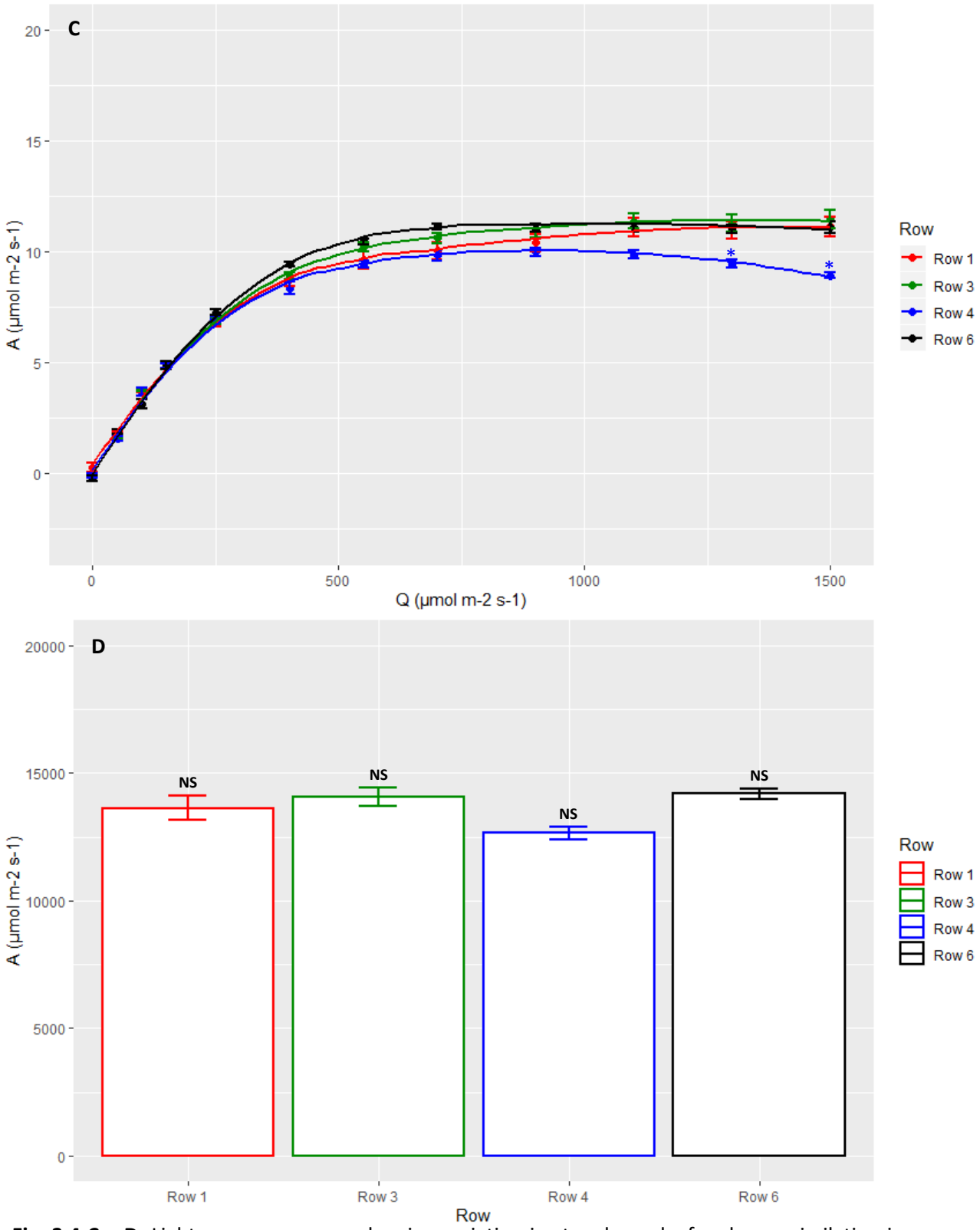

Fig. 3.4 C – D: Light response curves showing variation in strawberry leaf carbon assimilation in four different polytunnel rows in the mid-season (middle of fruiting period, early August). **C:** Afternoon measurements (3.30 PM – 7.30 PM). **D:** Integrated area under the curve of Fig 3.4.C. n = 4, error bars show standard error. * = significant difference between Row 4 and other rows (T-Test, n = 4, $p < 0.05$).

3.3.2 Effect of polytunnel row position on intrinsic water use efficiency

3.3.2.1 Strawberry leaves in Rows 1 and 4 have the greatest early season intrinsic water use efficiency but for different reasons

Intrinsic water use efficiency (WUEi) was calculated as follows:

$$WUEi = A * gs^{-1}$$

where A = assimilation and gs = stomatal conductance as described in Simkin *et al.*, 2015.

This provided a different metric by which to compare the capacity for carbon uptake between rows, since greater stomatal aperture will allow for greater conductance of atmospheric carbon into the leaf. Conversely, greater stomatal aperture is linked to greater water loss through evaporation and changes in *gs* are strongly correlated with responses to water stress and salt stress in strawberry (Grant *et al.*, 2012; Turhan and Eriş, 2007; Klamkowski and Treder, 2006; Li *et al.*, 2006; Orsini *et al.*, 2012; Johnson *et al.*, 2009). Interestingly, greatest WUEi has been found in mildly water stressed strawberry plants (field water at 50 % capacity), driven by a 71 % – 74 % reduction in *gs* (Ghaderi and Siosemardeh, 2011). Individuals with reduced stomatal aperture but greater, or similar, rates of assimilation to other individuals require less access to atmospheric carbon to synthesise a similar quantity of biomass than their counterparts (McAusland *et al.*, 2016), indicating greater photosynthetic efficiency and reducing evaporation of water from the leaf. This relationship between carbon and water has been demonstrated already in the results from 3.3.1.1. Understanding how crops can grow with reduced water use while maintaining yields is valuable for growers and for the environment, due to limited freshwater resources and reduced wastage.

In the early season (**Fig. 3.5.A – B**), comparing WUEi between rows reveals Rows 1 and 4 having the consistently highest ratio of assimilation to stomatal conductance in both the morning and afternoon, though this difference is not significant during the morning. It has

previously been shown that Row 4 exhibits some of the greatest assimilation rate between all rows and Row 1 some of the lowest in the early season (see **Fig. 3.3**). The high WUEi shown by both of these rows during the morning and afternoon must therefore be derived through different ways.

In the case of Row 1, a relatively low assimilation rate (compared to other rows) was offset by a relatively low *gs* value (**Fig. 3.6**). This allowed for a high *A*:*gs* ratio, giving a high *WUEi* and significantly greater *WUEi* than Row 3 at lower light intensities. Low *gs* is caused by reduced stomatal aperture – this in turn may explain the low assimilation rate seen in Row 1. In a study of seven Japanese strawberry varieties, net photosynthetic rate correlated strongly with stomatal conductance (Kanno *et al.*, 2022). The low stomatal conductance seen in Row 1 would therefore explain the low assimilation, since a smaller stomatal aperture would allow less carbon dioxide to enter the leaf.

Conversely in Row 4, a relatively high assimilation rate was sufficient to offset a greater *gs* value (although this *gs* was not significantly different to other rows) (**Fig. 3.6**), also giving a high *WUEi*. In the afternoon, Row 6 has a similar *WUEi* than Row 4 up to a given light intensity of ~650 µmol m^{-2} s^{-1} before declining at greater light intensities (see **Fig. 3.5.C**), although this not a significant change. As previously shown (see **Fig. 3.3.B**), the afternoon assimilation of Rows 4 and 6 is very similar. This shows that the stomatal conductance of Row 6 plants increases to accommodate greater assimilation, while Row 4 plants can perform the same rates of assimilation without a need to further open their stomata. To confirm if this pattern is truly occurring, further repeats would be valuable to identify significance. In strawberry grown at elevated CO_2 (720 ppm), where photosynthesis is heightened due to removal of atmospheric carbon as a limiting factor, plants responded to ozone stress through closing stomata. This response was not recorded in plants grown at ambient CO_2 (360 ppm) (Keutgen *et al.*, 1999). The difference in responses demonstrates that for strawberry plants performing photosynthesis

at an increased rate, stomatal closure (and thus, impaired carbon assimilation) is increased to reduce stress and water loss, since a sufficient rate of photosynthesis can still be performed with reduced stomatal aperture. Additionally, a correlation between increased PAR and decreased *gs* has previously been described in strawberry (Choi, 2021). Taken together, these findings would explain the relatively lower *gs* seen in Row 4, cement the hypothesis that Row 4 is capable of performing photosynthesis with greater efficiency and generate a new hypothesis that strawberry photosynthesis is strongly regulated by stomatal activity. This hypothesis is further supported by greatly increased *WUEi* in the afternoon (**Fig. 3.5.A**). At this time, *A* is greatly reduced but high *WUEi* indicates a relatively much lowered *gs*. This again demonstrates the stomatal limitation of carbon assimilation in strawberry leaves.

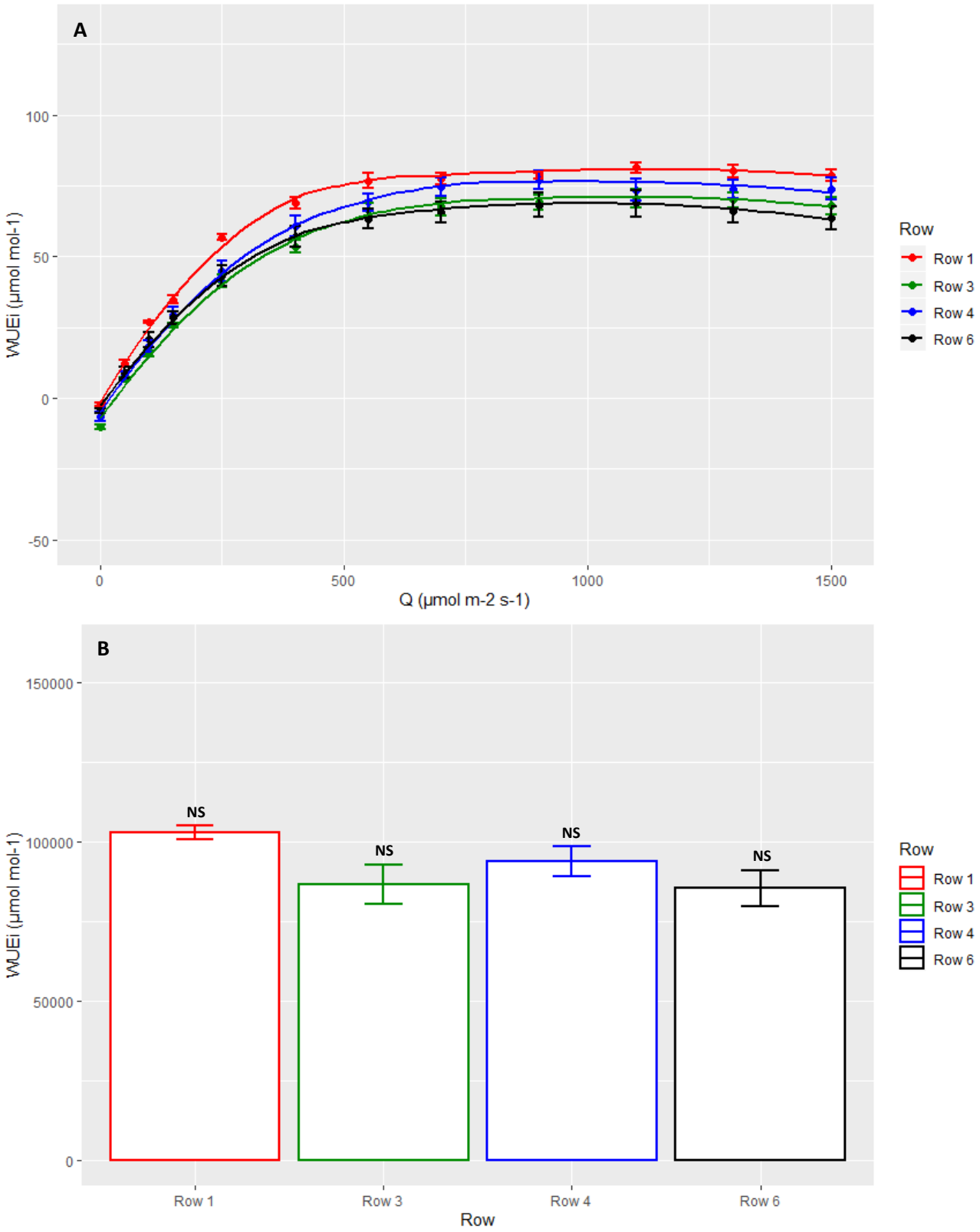

Fig. 3.5 A – B: Intrinsic water use efficiency at different light levels for strawberry leaves in four different polytunnel rows in the early season (beginning of fruiting, early/mid-June). **A:** Morning measurements (7 AM – 11 AM). **B:** Integrated area under the curve of Fig 3.5.A. n = 4, error bars show standard error.

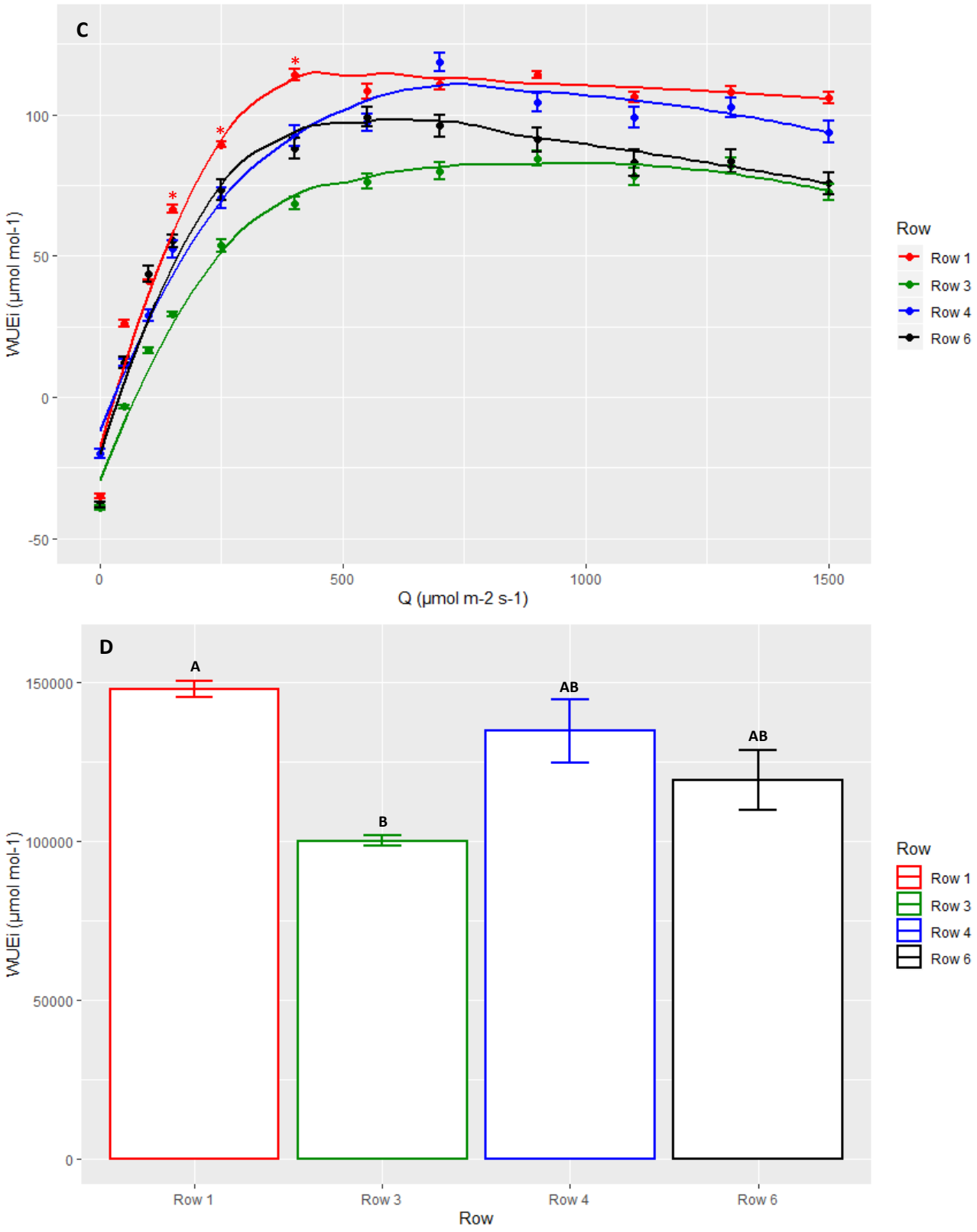

Fig. 3.5 C – D: Intrinsic water use efficiency at different light levels for strawberry leaves in four different polytunnel rows in the early season (beginning of fruiting, early/mid-June). **C:** Afternoon measurements (3.30 PM – 7.30 PM). **D:** Integrated area under the curve of Fig 3.5.C. n = 4, error bars show standard error. * = significant difference between Row 1 and Row 3 (T-Test, n = 4, $p < 0.05$).

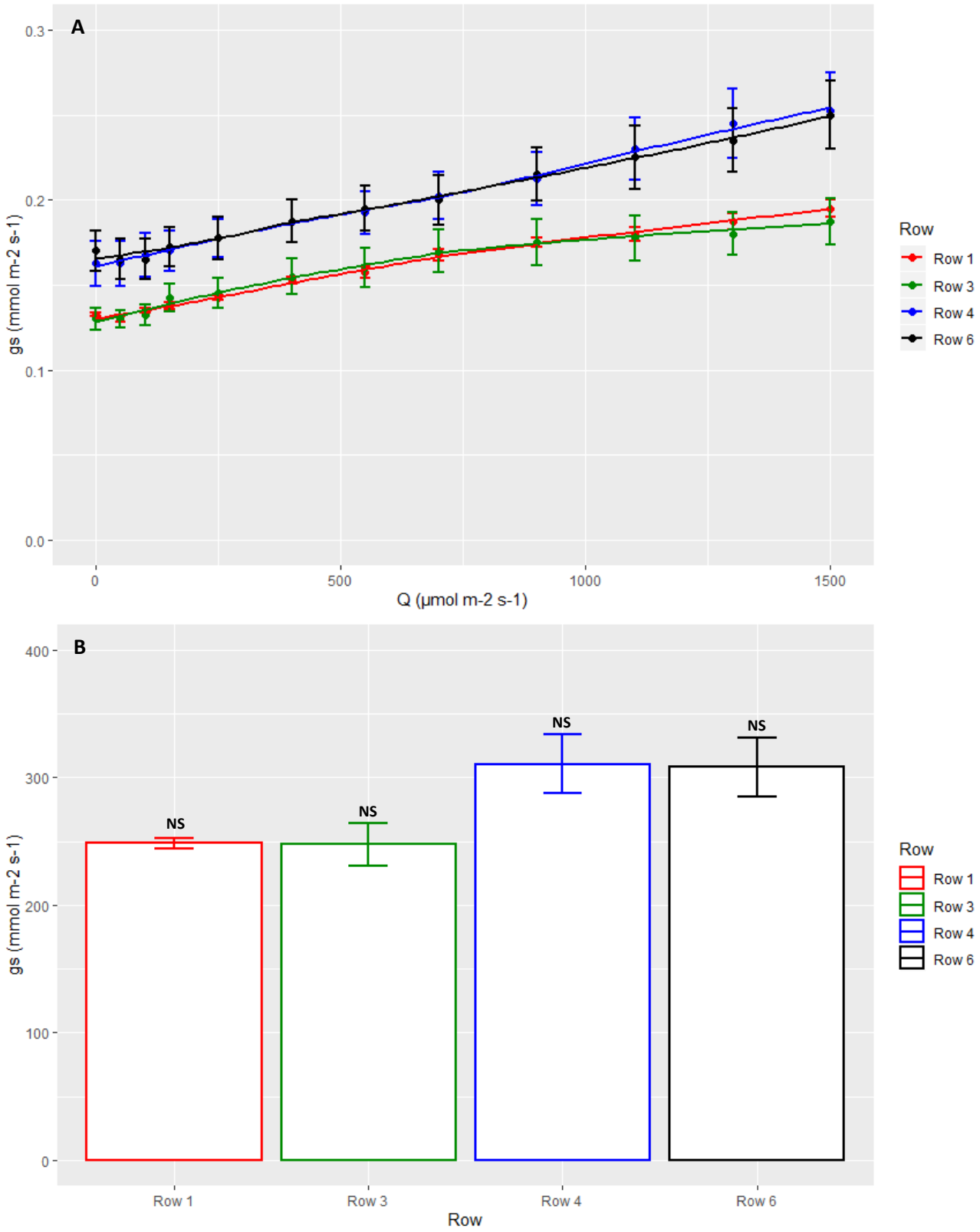

Fig. 3.6 A – B: Stomatal conductance at different light levels for strawberry leaves in four different polytunnel rows in the early season (beginning of fruiting, early/mid-June). **A:** Morning measurements (7 AM – 11 AM). **B:** Integrated area under the curve of Fig 3.6.A. n = 4, error bars show standard error.

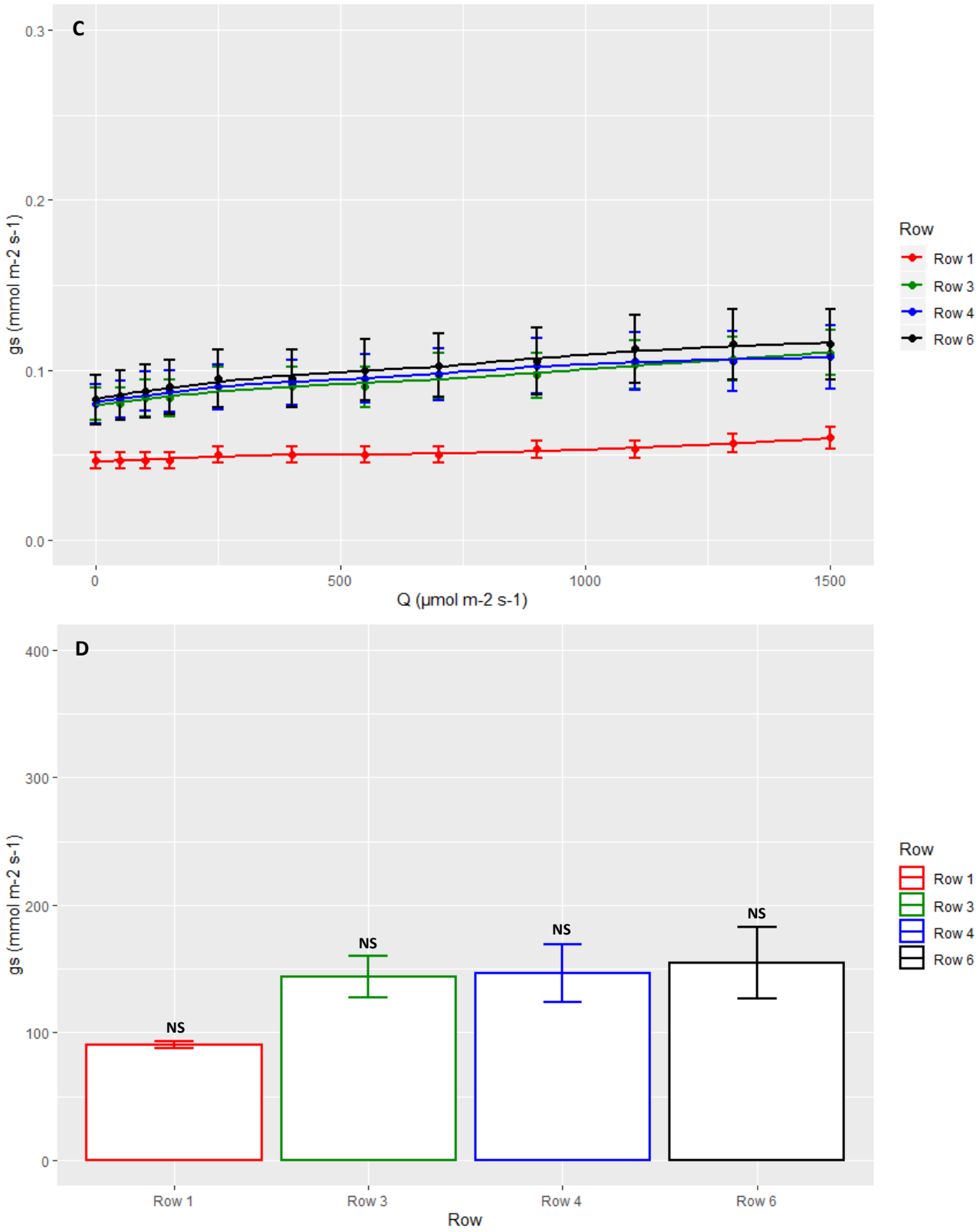

Fig. 3.6 C – D: Stomatal conductance at different light levels for strawberry leaves in four different polytunnel rows in the early season (beginning of fruiting, early/mid-June). **C:** Afternoon measurements (3.30 PM – 7.30 PM). **D:** Integrated area under the curve of Fig 3.6.C. n = 4, error bars show standard error.

3.3.2.2 Strawberry leaves in 4 have the greatest mid season intrinsic water use efficieny

Measurements of *WUEi* were repeated during the mid-season (see **Fig. 3.7**). Despite a largely reduced capacity for assimilation in Row 4 during this time (as displayed in **Fig. 3.4**), Row 4 still exhibited a significantly high *WUEi*, especially during the afternoon when its capacity for carbon assimilation was at its lowest. It is therefore apparent that Row 4 has greater *WUEi* due to reduced stomatal conductance – this reduction was significant at higher light levels (> 700 µmol m^{-2} s^{-1}, T-Test, $p < 0.05$). While this has a detrimental effect on Row 4 carbon assimilation, the relatively similar assimilation during the morning and at lower light levels in the afternoon (when light is already becoming more disperse, reducing light intensity and PAR), demonstrate that Row 4 is able to operate at a greater photosynthetic rate while maintaining a smaller stomatal aperture, indicating greater photosynthetic efficiency. This further lends credence to the concept discussed above; that strawberry stomatal aperture may heavily regulate photosynthesis, such that maximal photosynthetic capacity is limited (see 3.3.2.1 for full discussion).

The *WUEi* of Row 1 plants is also curious here. In the early season, Row 1 plants exhibited the highest *WUEi* (see **Fig. 3.5**) but by the mid-season the *WUEi* is lower than any other row. At this time, Row 1 photosynthetic carbon assimilation was much more similar to other polytunnel rows than in the early season (see **Fig. 3.3** and **Fig. 3.4** for comparison). This may explain the low *WUEi* seen. Greater stomatal aperture to increase uptake of atmospheric carbon would increase assimilation rate at the cost of leaf water evaporation (i.e. increased *gs*). This change in activity may have been driven by cooler mid-season temperatures, reducing the evaporation of water from the leaf and making the increase in stomatal aperture seen more energetically viable, despite a relative reduction in *WUEi*.

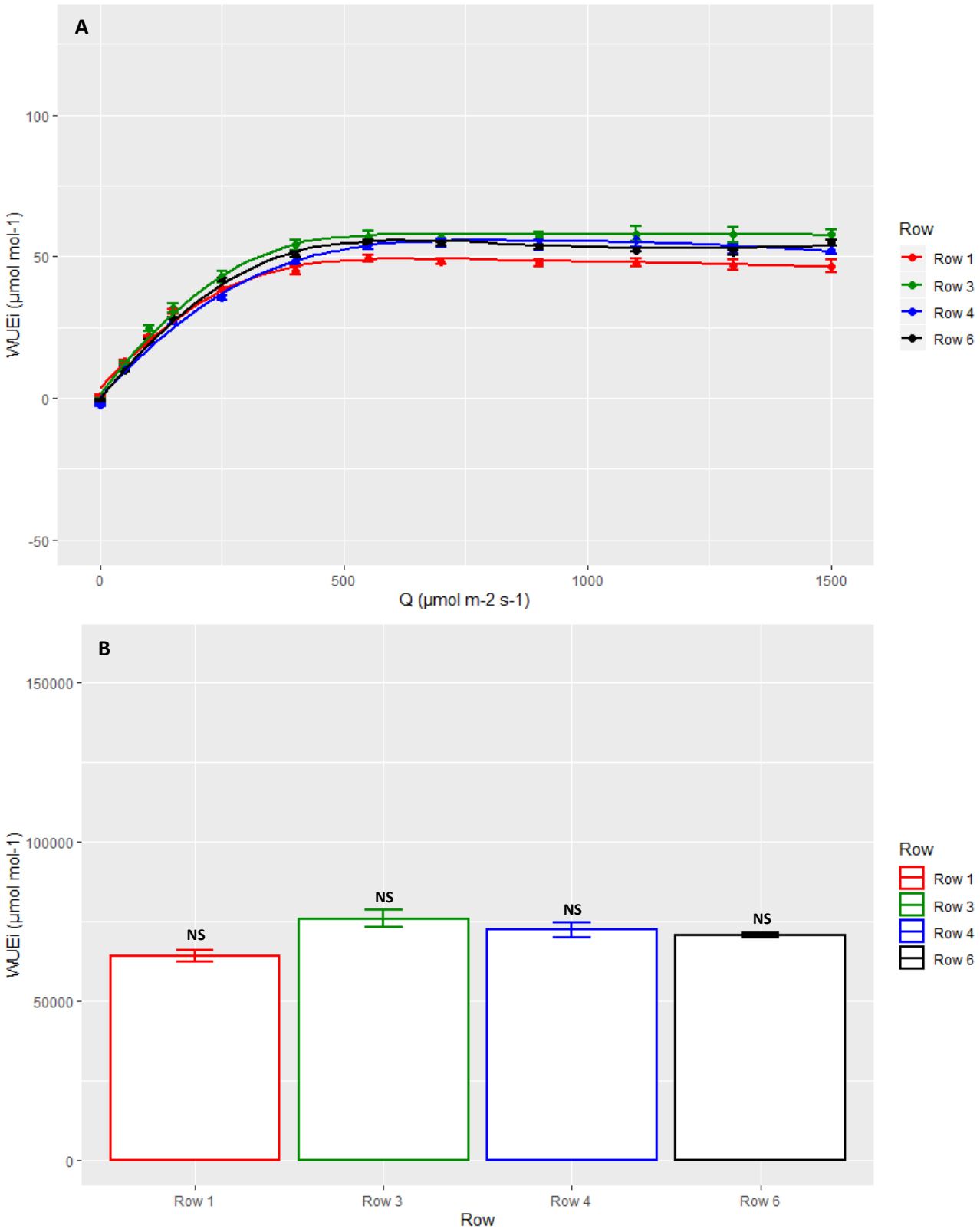

Fig. 3.7 A – B: Intrinsic water use efficiency at different light levels for strawberry leaves in four different polytunnel rows in the mid-season (middle of fruiting period, early August). **A:** Morning measurements (7 AM – 11 AM). **B:** Integrated area under the curve of Fig 3.7.A. n = 6, error bars show standard error.

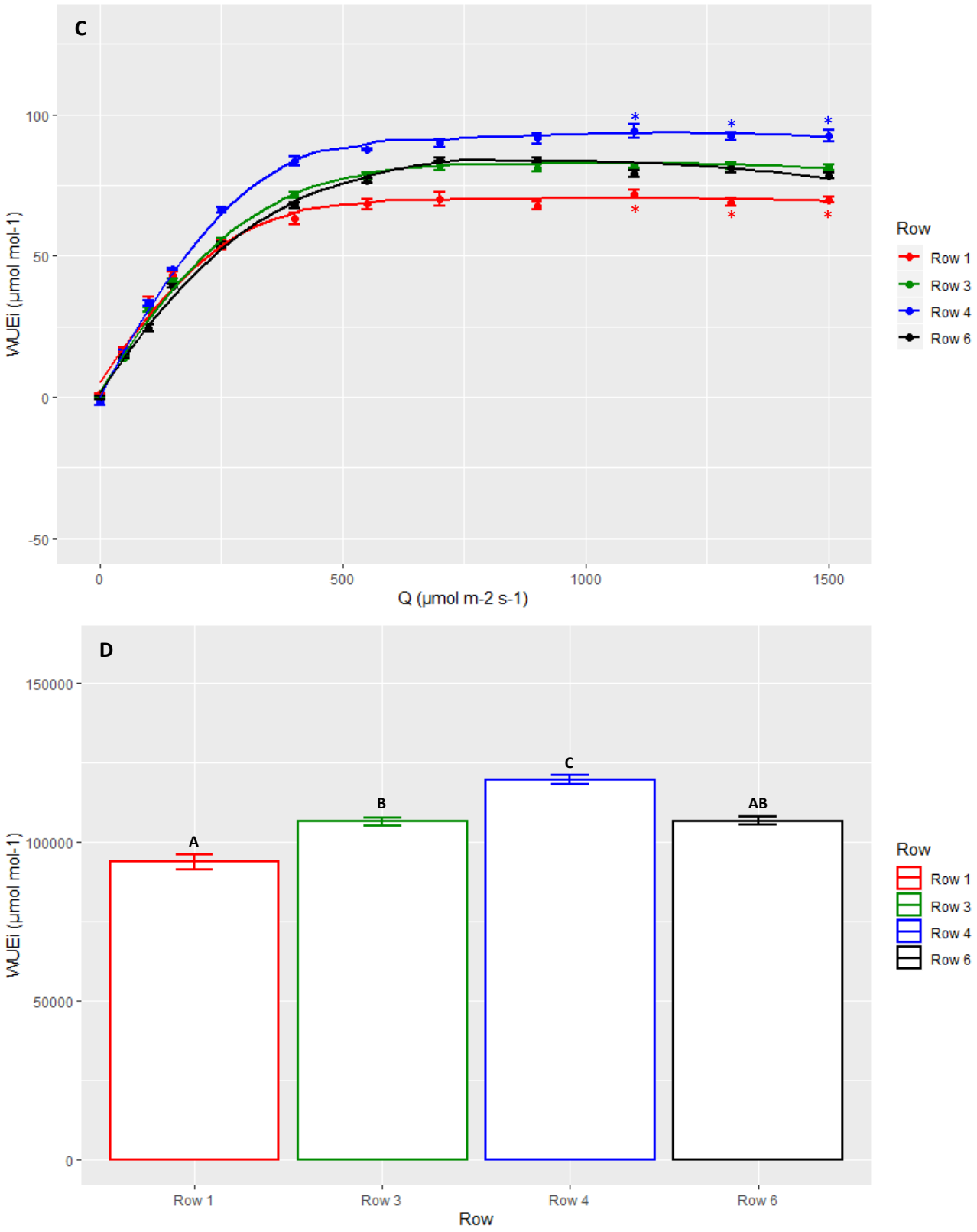

Fig. 3.7 C –D: Intrinsic water use efficiency at different light levels for strawberry leaves in four different polytunnel rows in the mid-season (middle of fruiting period, early August). **C:** Afternoon measurements (3.30 PM – 7.30 PM). **D:** Integrated area under the curve of Fig 3.7.C. n = 6, error bars show standard error. * = significant difference between Rows 1/4 and Rows 3/6, colours match rows (T-Test, n = 6, $p < 0.05$).

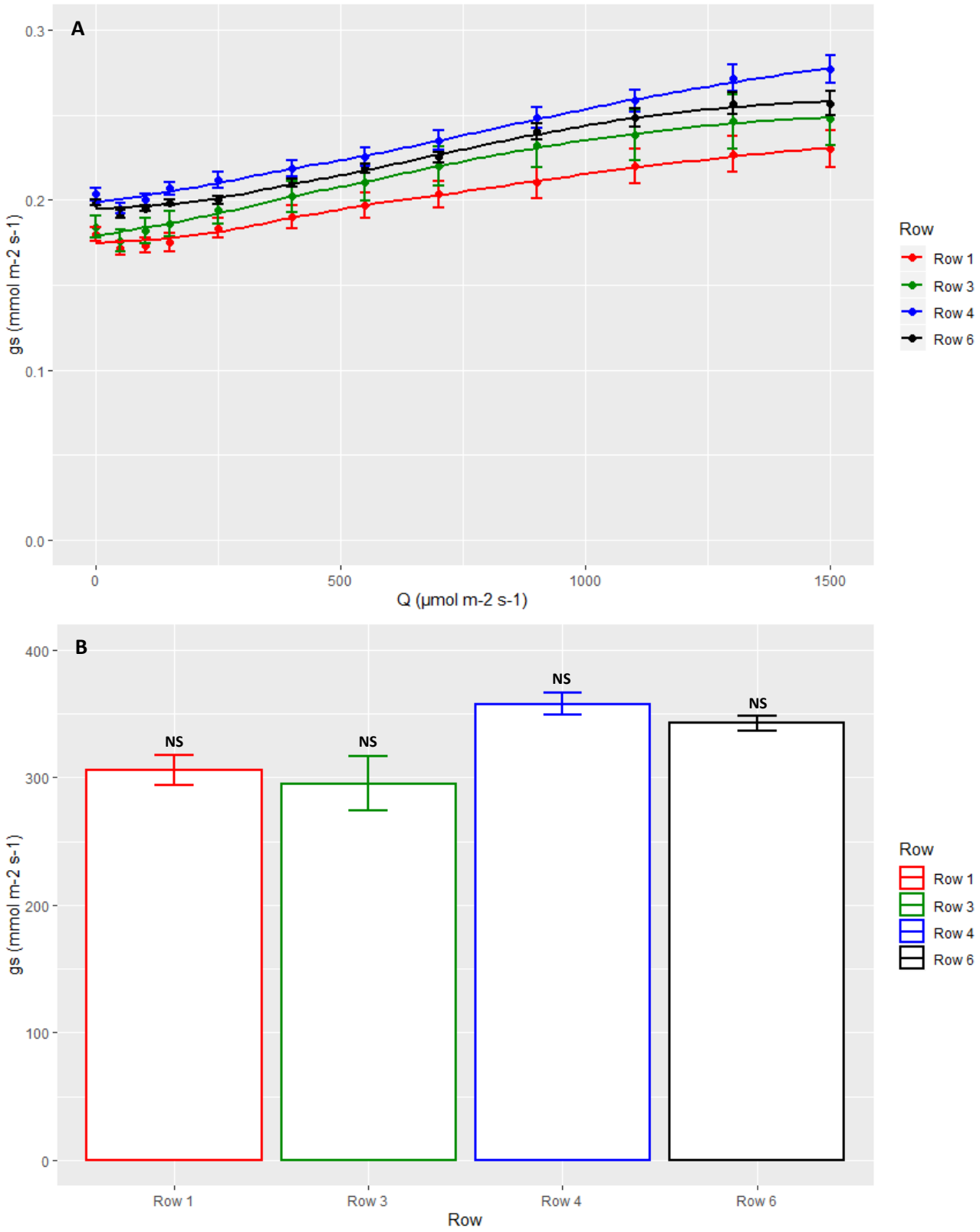

Fig. 3.8 A – B: Stomatal conductance at different light levels for strawberry leaves in four different polytunnel rows in the mid-season (middle of fruiting period, early August). **A:** Morning measurements (7 AM – 11 AM). **B:** Integrated area under the curve of Fig 3.8.A. n = 6, error bars show standard error.

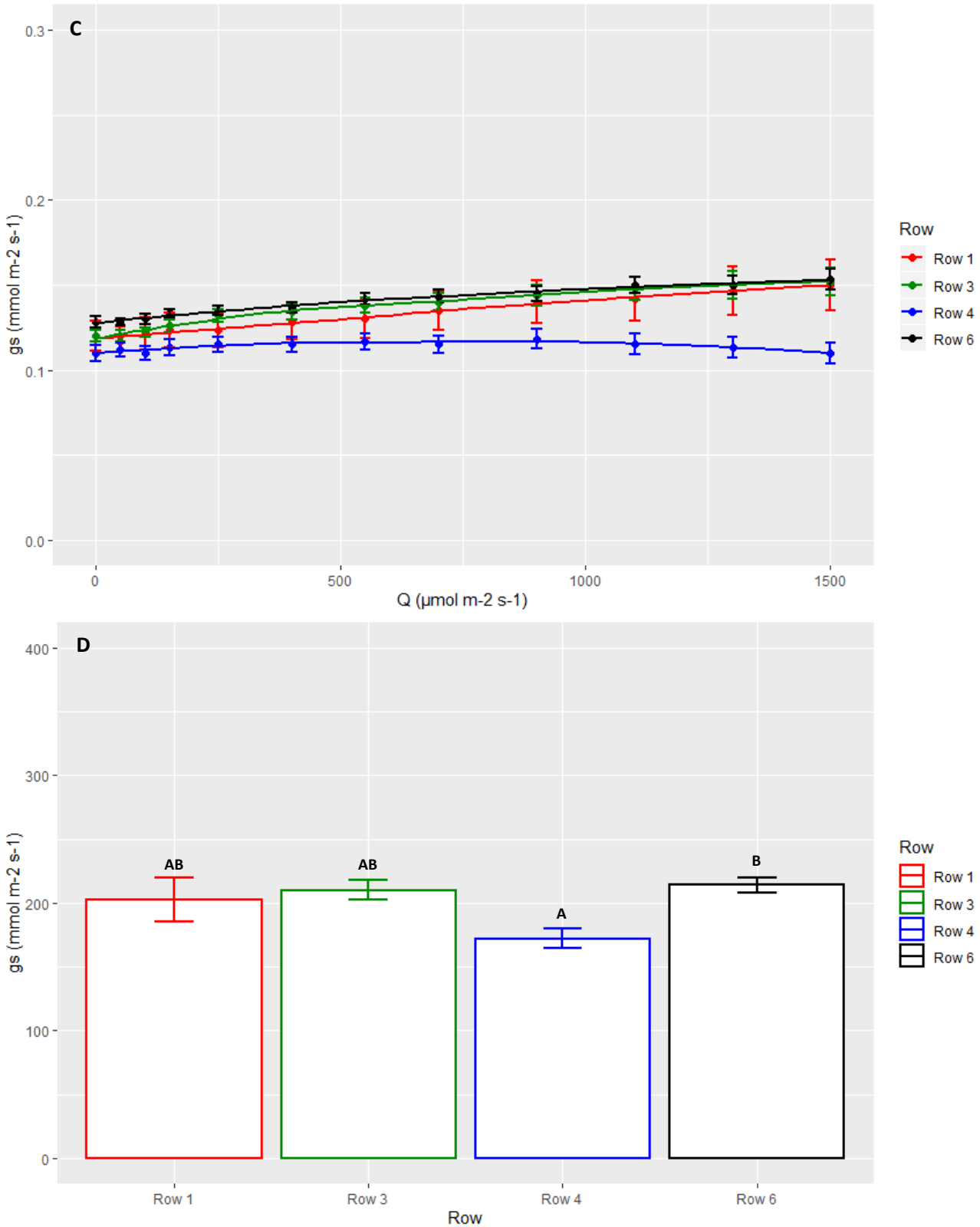

Fig. 3.8 C –D: Stomatal conductance at different light levels for strawberry leaves in four different polytunnel rows in the mid-season (middle of fruiting period, early August). **C:** Afternoon measurements (3.30 PM – 7.30 PM). **D:** Integrated area under the curve of Fig 3.8.C. n = 6, error bars show standard error.

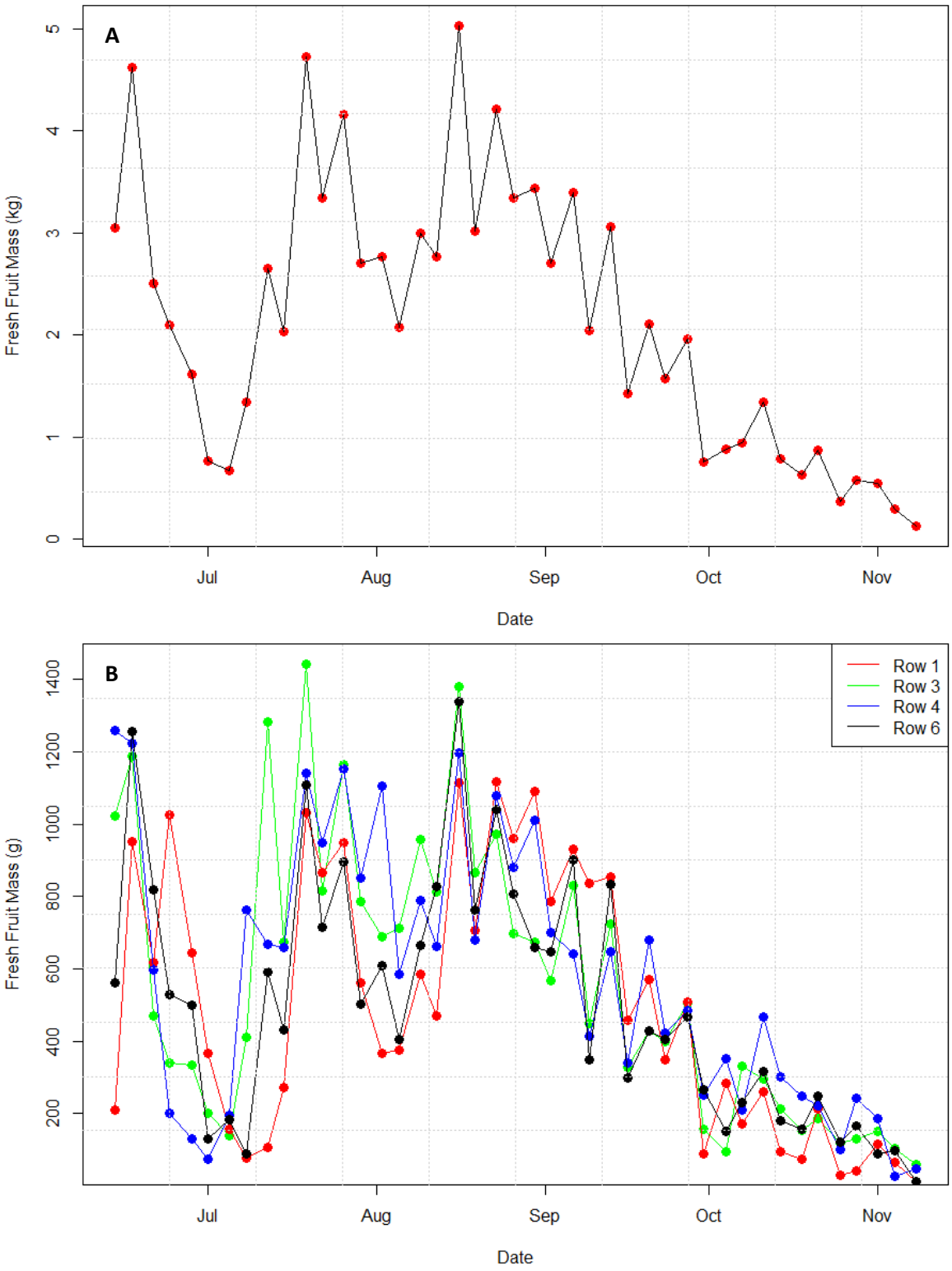

Fig. 3.9: Patterns of fresh fruit yield across the growing season. **A:** Total fresh fruit yield across the growing season. **B:** Fresh fruit yield across the growing season by row.

3.3.3 Effect of polytunnel row position on fruit yield

3.3.3.1 Fruit yield shows three major production peaks across the growing season but individual rows show marginally different patterns

Strawberry fruits were picked from 32 plants per row across the growing season twice a week (Mondays and Thursdays). Fruit mass, class and number were recorded. This identified three flushes of production across the season in mid-June, mid-July and mid-August (see **Fig. 3.9.A**). This is later than is typical of Malling Centenary (50 % harvest date in a 2012 trial was 4th June (Whitehouse *et al.*, 2017)) , however a cold spring delayed development and pushed the season back. This pattern can also be seen on an individual row level (see **Fig. 3.9.B**). In line with the photosynthetic patterns seen, Row 4 had greater production at the beginning of the season than any other row, indicating the use of greater assimilated carbon in developing fruit biomass and further highlighting the established link between strawberry fruit yield and increased photosynthesis (Choi *et al.*, 2016). Row 1 lagged behind the patterns seen in the other rows by approximately a week for the first month before catching up. This may reflect the lower photosynthetic assimilation in Row 1, as greater photosynthesis has previously been demonstrated to accelerate plant development (Simkin *et al.*, 2017) and lower availability of PAR has been shown to delay floral initiation (Thomas and Raper, 1979).

3.3.3.2 Row 4 shows the greatest fresh fruit yield overall but central rows and edge rows show similar fruit yields

Total fruit yield was calculated by adding masses and numbers of picked fruits. This also provided a cumulative yield to again demonstrate fluctuations in fruit flushes (see **Fig. 3.10**). Row 4 demonstrated the highest total class 1 yield, 3 % greater than Row 3 and 17 % – 20 % greater than Rows 1 and 6. While Row 4 exhibited the highest fruit yield, this was only marginally greater than Row 3. Similarly, while Row 6 exhibited greater fruit yield than Row 1, the final difference in cumulative yield was similar. This indicates segregation of yield across the tunnel, where the central rows yield greater numbers and masses of fruits and the external rows less. One possible explanation for this is greater PAR made available to the central rows (as discussed in 3.3.1.1). Condori *et al.*, (2017) found that 41 % of variation in strawberry yield along a polytunnel length could be explained by fluctuations in light and temperature and at sub-optimal temperatures (optimal air temperature was found to be 26.8 °C) increased PAR strongly correlated with greater yield. The greater light intensities towards the centre of the tunnel may have created a more favourable microclimate for growth, leading to segregation of yield characteristics between inner and outer rows.

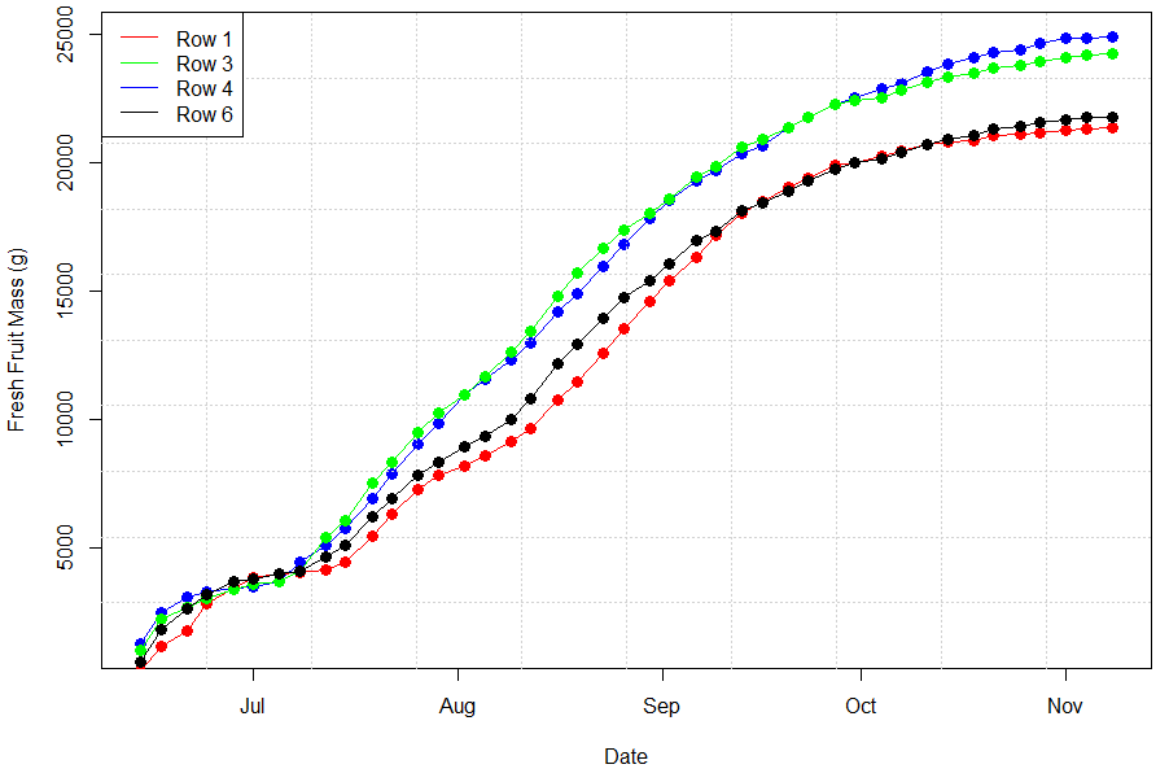

Fig. 3.10: Cumulative Class 1 fresh fruit yield of strawberry fruit in different polytunnel rows.

3.3.3.3 Increased fruit yield is derived from increased fruit number and Row 4 exhibits greatest total fruit number

Total fruit number was recorded alongside fruit mass (see **Fig. 3.11**). Row 4 was found to have the greatest fruit number and Row 1 the lowest. This correlates with recorded differences in photosynthesis between rows. It should also be noted however that Row 4 had the smallest fruits, perhaps indicating a greater rate of ripening or sink limited growth while Row 1 had the largest fruits; this may go some way to explaining the segregation patterns in fruit yield displayed in **Fig. 3.10**.

This is an expected relationship. Light is a driver of floral initiation (Wang *et al.*, 2020) and it has previously been demonstrated that runner propagated everbearing strawberries produce higher numbers of flower trusses when grown under supplemental lighting (Maas and Cathey, 1987). It is likely then that the rows which receives the highest level of PAR (i.e. Rows 3 and 4) would see the greatest number of fruits due to increased floral initiations. It should be noted that this result does not correlate with historical reported data of greater yield exclusively in Row 4 relative to fruit yield of the other rows. This may be related to an as yet unstudied edge effect at the north and south ends of the tunnel, since fruit was collected exclusively from the centre of the tunnel to remove any potential error. Again, the work carried out by Condori *et al.*, (2017) may explain this due to sub-optimal temperatures being reached at the tunnel ends and leading to a greater effect of PAR on yield.

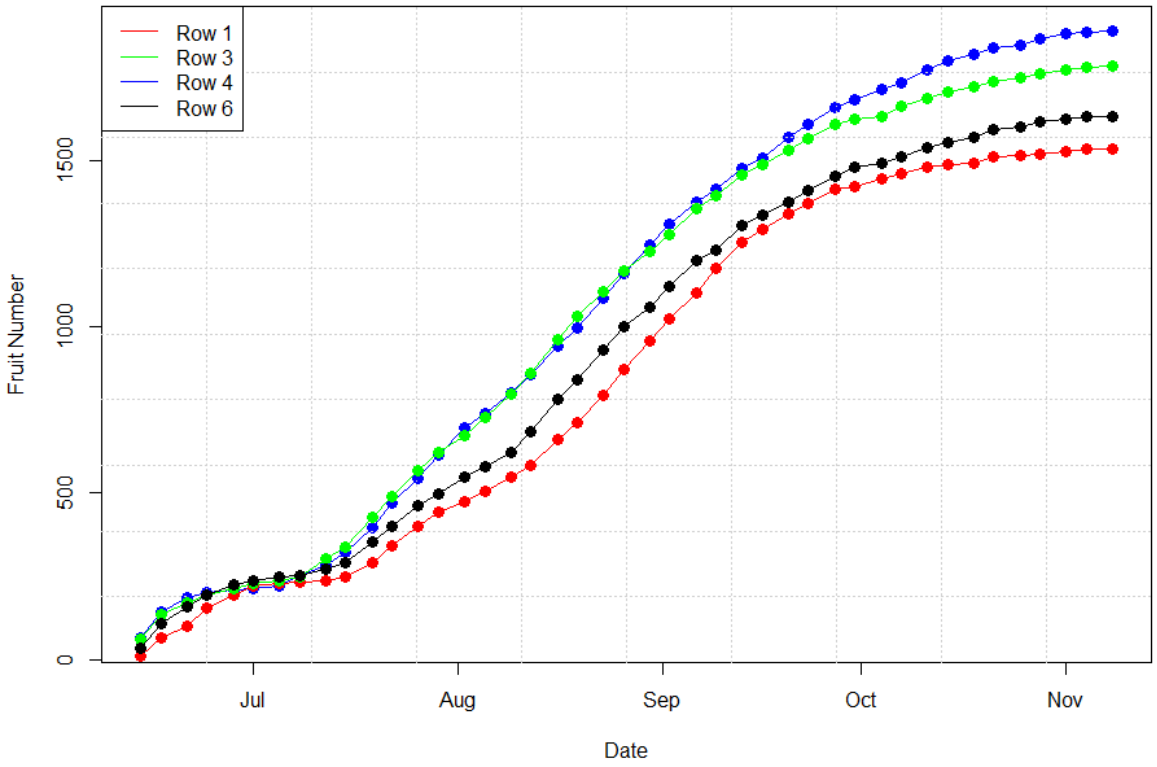

Fig. 3.11: Cumulative Class 1 fruit number of strawberry fruit in different polytunnel rows.

3.3.3.4 Proportion of Class 1 fruit decreases from east to west across the polytunnel while proportion of waste fruit increases

Fruits were classed according to size, shape, colour and condition (see Materials and Methods section 2.2.3 and **Fig. 3.12**). Row 1 exhibited the greatest proportion of Class 1 fruit (86.7 %) while Row 6 exhibited the lowest proportion (82.7 %). Conversely, Row 6 exhibited the greatest proportion of waste fruit (7.1 %) while Row 1 exhibited the lowest proportion of waste fruit (4.6 %). Rows 3 and 4 had similar proportions of Class 1, Class 2 and waste fruits. The high proportion of Class 1 fruit in Row 1 may be reflective of the lower fruit number identified in this row. Due to fewer fruits being present, a greater proportion of assimilated carbon may be available to these fruits for growth, resulting in larger secondary and tertiary fruits that could reach Class 1 size. The lower proportion of Class 1 fruit in Row 6, and higher percentage of waste fruits, could be explained by inaccessibility for pollinators (Howard *et al.*, 2021), reduced ability to reach full canopy activation in the morning due to polytunnel position and limited PAR reducing photosynthetic rate.

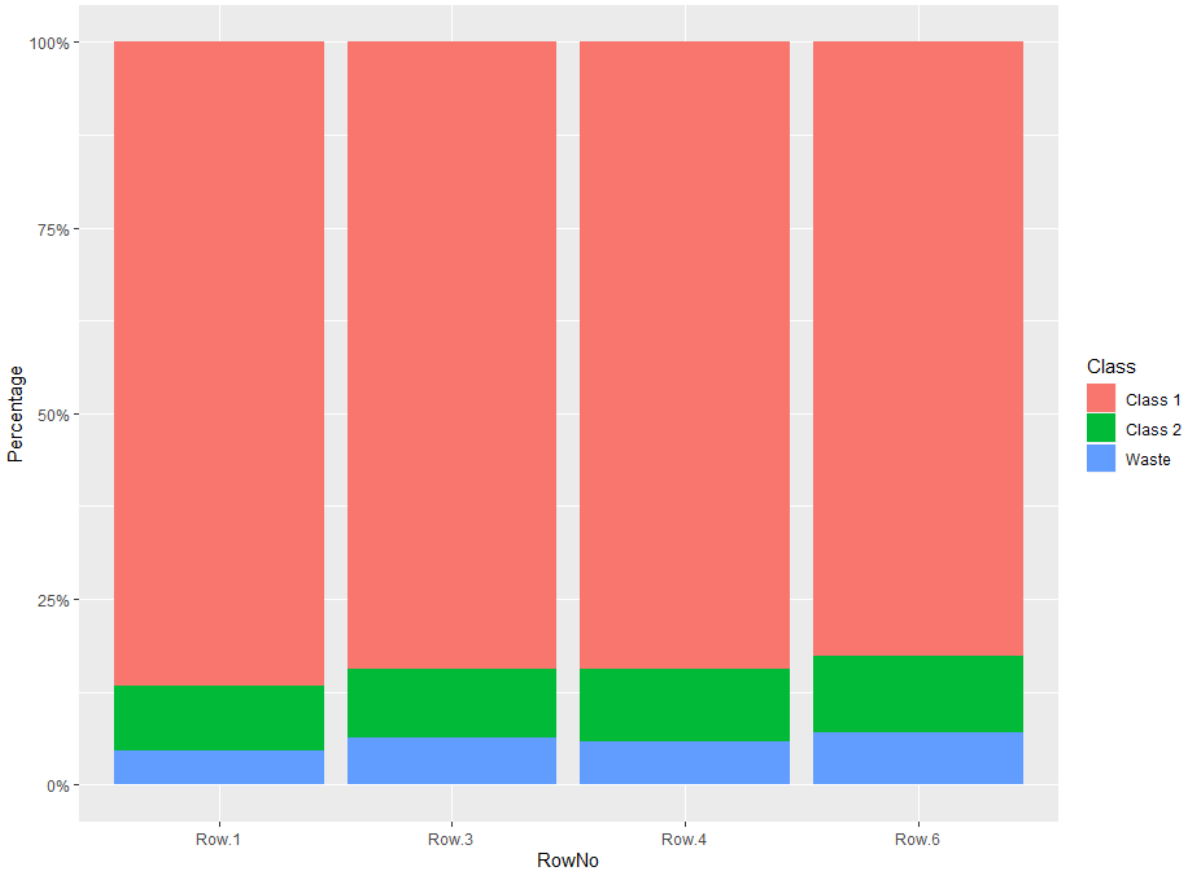

Fig. 3.12: Distribution of fruit classes between polytunnel rows.

3.3.3.5 Internal edges of Rows 4 and 6 exhibit significantly greater fruit yields

Due to the planting pattern within coir bags (see **Fig. 2.2**), individual plants are spaced in such a way that the lower canopy and most trusses fall along one side of the row. Comparing the yield of plants grown on the west or east side of the row revealed significant differences in the westernmost rows (Rows 4 and 6, see **Fig. 3.13**). This unusual pattern may be explained by radiation of absorbed heat from the row structure creating a more favourable microclimate for larger fruit development. This may also explain the pattern across the tunnel. Light entering the polytunnel from the west would heat the interior of the eastern side of the tunnel and the exterior of the western side, causing the eastern interior metal framework to heat more than the western interior. This would create favourable conditions for fruits growing on the eastern edge (as seen in Rows 1 and 6), allowing the microclimate around those plants to remain in the optimal range for yield for longer (previously identified as 26 – 34 °C (Carlen *et al.*, 2009)).

In the case of Row 4, the eastern edge is on the central, exposed part of the tunnel (see **Fig. 2.2**), allowing for greater heat loss via convection. This temperature differential could be further aided through heat radiation from the metal structure of Row 5. Given that strawberry yield at high-light is limited by temperature (Rivero *et al.*, 2021) and Row 4 receives greater PAR than other polytunnel rows, this could explain the relatively higher yield of the less exposed, western edge. This, however, is just conjecture – further study is needed to explore temperature microclimates in field environments to better understand fluctuations in yield.

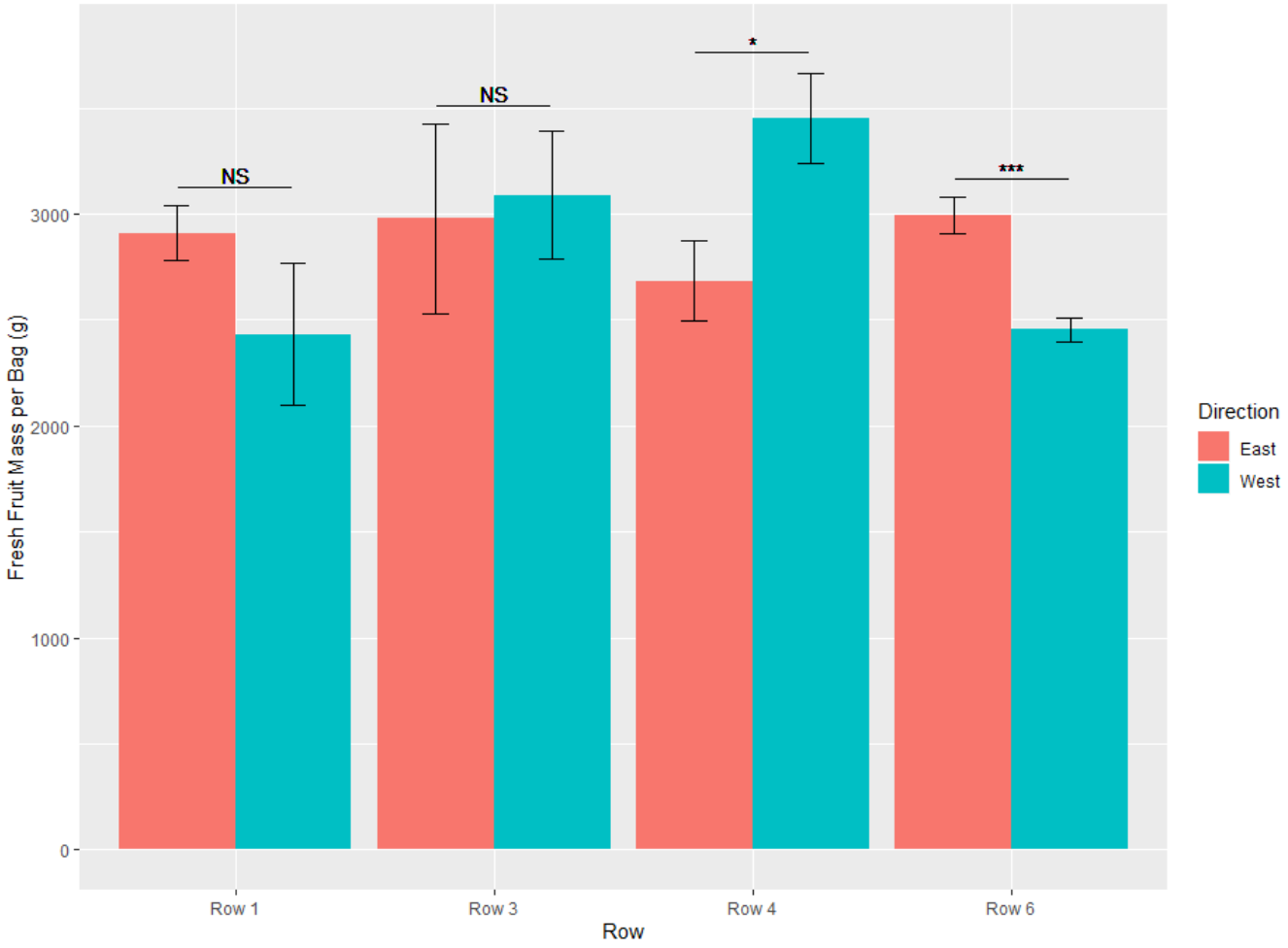

Fig. 3.13: Class 1 fresh fruit yield of east and west facing plants in coir bags. n = 4, error bars show standard deviation. * = $p < 0.1$, *** = $p < 0.01$. Significance tested using Welch's t-test for unequal variances.

3.4 Summary

Strawberry photosynthetic characteristics vary significantly across a polytunnel. This is correlated with differences in yield between polytunnel rows. Row 4 demonstrates the greatest total rates of photosynthetic carbon assimilation across seasons and Row 1 the lowest. This is reflected in Row 4 producing the greatest numbers and final yield of Class 1 strawberry fruits, whereas Row 1 produced the lowest numbers and final yield of Class 1 fruits. Strawberry photosynthesis appears to be strongly limited by stomatal activity. Under conditions favourable to photosynthesis (i.e. high PAR), opening of stomata is limited to conserve water over opening more to increase access to atmospheric carbon (since sufficient photosynthesis is already being performed with the carbon available). Peaks of temperature in the centre of the polytunnel and between rows may be responsible for generating yield differences between the central and edge rows of the tunnel, as well as between plants grown on more or less sheltered edges of the rows themselves. This reveals temperature as another key regulator of yield, though if this has an effect on strawberry photosynthesis is not explored in this chapter.

Future work should explore differences across the east-west axis at the north and south ends of a polytunnel, since edge effects at polytunnel ends have not been explored in this work and may cause greater row-to-row variation. Responses of different cultivars should also be considered, since significant cultivar-to-cultivar variation in strawberry *gs* has already been identified (Grant *et al.*, 2012; Grant *et al.*, 2010). The effect of different temperatures on strawberry yield needs further study in a field environment to prevent chamber effects and to better understand how fluctuations in microclimate temperature affects yield. Finally, the effect of supplemental lighting within a polytunnel should be explored. It is apparent that, even with stomatal limitation, greater PAR is correlated with increased photosynthesis and yield and if this could be introduced into a polytunnel, it could greatly stimulate yields.

To conclude:

- Row 4 received the greatest PAR in April but segregation in received PAR was ultimately not between Row 4 and all other rows but between central and external rows.
- Row 4 had greatest capacity for photosynthetic carbon assimilation at high light in the mornings of the early season but this effect was lost in the afternoons and the mid-season. The capacity for photosynthetic carbon assimilation under high light did not come at a cost of water use efficiency.
- PAR was highly correlated with fruit number and yield, with central rows producing greater numbers of fruits than external rows.

Chapter 4 – Transformation of Cultivated Strawberry with Photosynthetic Genes of Interest

4.1 Introduction

As previously discussed in Chapter 1, a large body of work has been published exploring how photosynthesis can be manipulated to increase photosynthetic efficiency in model and crop species, with concomitant increases in biomass and yield seen. Recent research has studied the use of stacking rate-limiting enzymes of photosynthesis to effect larger increases. This chapter aims to replicate this method in strawberry. It has already been identified that increased photosynthesis is correlated with increased yield returns in strawberry (see Chapter 3). It follows that, by increasing photosynthetic efficiency through manipulating strawberry genetics, that greater yielding lines could be generated. This chapter examines the work undertaken to successfully genetically modify strawberry, a task that provides unique, species-specific challenges, from gene selection through to preparation for genotypic and phenotypic analysis. To the author's knowledge, this would be the first time genetic modification of strawberry has been used to increase photosynthetic efficiency.

The hypotheses to be considered in this chapter are as follows:

- Exposing strawberry leaf material to transgenic agrobacterium will produce transgenic callus.
- Transgenic callus will develop into plants with fully differentiated roots, shoots and crowns.
- Transgenic plants will have detectable expression of transgenes.

4.2 Methods

Materials and methods were carried out as described in Chapter 2, sections 2.3.1 – 2.5.1.

4.3 Results and Discussion

4.3.1 Plasmid Generation

4.3.1.1 Theoretical Background

To begin this work, genes were selected for study. Sedoheptulose-1,7-bisphosphatase (SBPase) was selected due to its extensive previous study that has shown consistent improvements to photosynthetic efficiency across model and crop species (Lefebvre *et al.*, 2005; Driever *et al.*, 2017; Simkin *et al.*, 2015). SBPase is a highly regulated enzyme. It is activated during illumination of green tissue via the ferrodoxin/thioredoxin system and an increase in the concentration of stromal magnesium ions and catalyses a branch point within the CBC (**see Fig. 1.2**) (Raines *et al.*, 2000). This high level of regulation at a critical point of the cycle gives it a large degree of control over CBC flux (as discussed in 1.2.2). A second gene was also selected to study the effect of gene stacking to further increase photosynthetic efficiency. Adenosine diphosphate glucose pyrophosphorylase (AGPase) was selected due to the possibility of sink-limited growth. Overexpression of AGPase was hypothesised to increase phloem loading from excess transitory starch to help overcome this limitation (1.5.3 for further discussion of AGPase). Recent work has revealed that everbearing strawberry, as used in this work, does in fact experience source-limited growth (Sønsteby *et al.*, 2021), a result supported by hypotheses discussed in Chapter 3, and so it would be expected that increasing source strength alone by improving photosynthetic efficiency would still lead to yield returns.

4.3.1.2 Initial cloning efforts failed to produce the desired product

RNA was extracted from aerial tissue of *A. thaliana* and cDNAs generated via reverse transcription. *A. thaliana* genetic material was selected to minimise transgene silencing – a phenomenon in which a transformed individual actively downregulates expression of the transfected gene (Vaucheret *et al.*, 1998). From this cDNA, the cDNAs coding for the SBPase gene (AT3G55800) and AGPase gene (large subunit APL1, AT5G19220) were amplified. SBPase cDNA was 1182 nt and AGPase cDNA was 1596 nt. Amplified cDNAs were purified with this result being quantified via gel electrophoresis (**Fig. 4.1.A**). Primers were designed such that a CACC base overhang was present on the 5' end of the forward primers. This was to enable easier, one-directional cloning using the PENTR-D TOPO construct (Thermofisher, see 2.3.2 for construct map).

Following cloning of these fragments into the pENTR-D TOPO construct, *E. coli* were transformed with the product, grown on kanamycin selection (resistance gene present in the vector backbone) and colony PCR used to confirm insertion (visualised via gel electrophoresis in **Fig. 4.1.B-C**). Smearing on these two gels was attributed to overloading the gel with bacterial DNA. Despite this, the gels revealed an apparently successful transformation with faint bands present at 1182 bp and 1596 bp as expected for SBPase and AGPase respectively, although fewer colonies than expected grew on the plate. DNA was extracted from E. coli and used to prepare minipreps for sequencing. Sequencing revealed negative results (Eurofins, London, UK). This confirmed that the transformation was unsuccessful, explaining the low colony count. The false positive gel result was attributed to contamination of the bacterial DNA sample and colony formation attributed to some bacteria having natural kanamycin resistance or uneven distribution of kanamycin through the agar. This approach was attempted several times using three separate kits from the same company with no success. On investigation, it was revealed

there was a supplier issue that may have affected kit performance. As such, a different method was sought.

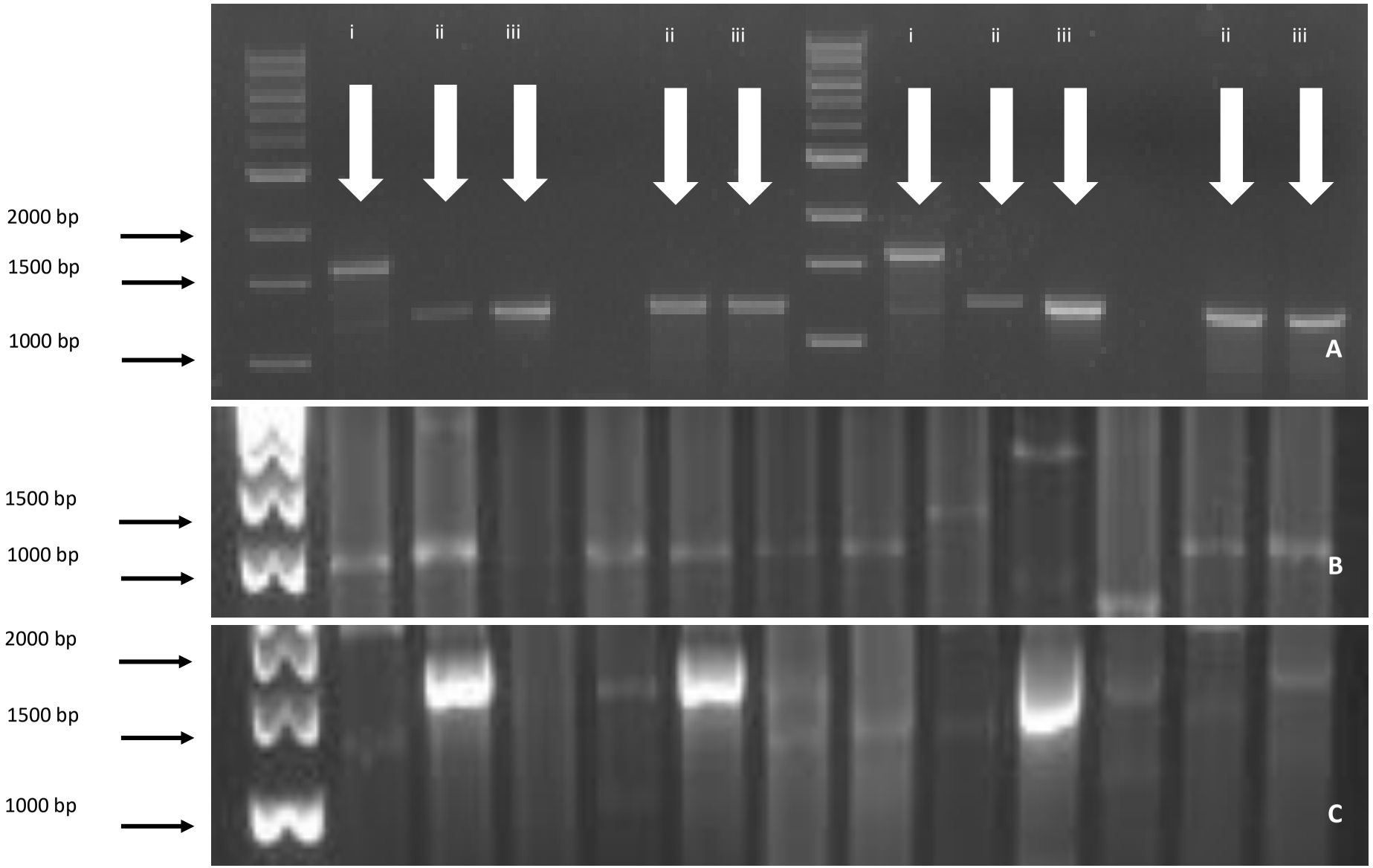

Fig. 4.1: Gel electrophoresis scans showing progression of cloning experiments. **A:** Purified cDNAs obtained from reverse transcription of target gene mRNA; **i:** AGPase; **ii:** SBPase; **iii:** SBPase positive control. **B:** SBPase CDS fragments amplified from transformed *E. coli*. **C:** AGPase CDS fragments amplified from transformed *E. coli.*

4.3.1.3 Cloning of constructs using Golden Gate Assembly

Following the unsuccessful attempts at cloning using the PENTR-D TOPO kit, construct generation was carried out at the University of Essex using modules borrowed from their library (see 2.3.5 for a list of the modules used).

Single gene constructs containing AtSBPase and AtAGPase coding sequences (CDS) were donated by the University of Essex. The SBPase construct contained the hygromycin resistance gene for plant selection with the SBPase CDS downstream of the cauliflower mosaic virus 35S (CaMV 35s) promoter (**Fig. 4.2.A**). The CaMV 35s promoter is regularly used in genetic technologies for its high level, constitutive expression (Kay *et al.*, 1987) and is found in greater than 80 % of constructs used in the generation of genetically modified plants (Bak and Emerson, 2020). The AGPase constructs contained the AGPase large subunit 1 (APL1) CDS downstream of the 35s promoter and RuBisCO small subunit promoter (RbcS) with hygromycin and glufosinate resistance genes present respectively (**Fig. 4.2.B – C**). The RbcS promoter was selected for its high expression level, specificity to expressing only in green tissue and its activity in the nucleus allowing for green tissue specific expression outside of the chloroplast (Manzara and Gruissem, 1988). It was necessary to test AGPase under this promoter, since constitutive expression of AGPase causes excessive starch synthesis to the point of callus death during the regeneration step of the transformation process (Stark *et al.*, 1992). Collectively, this suite of constructs would enable study of pleiotropic single gene overexpression effects with the possibility of comparing tissue specific and non-specific expression.

Multigene constructs containing AtSBPase downstream of the 35S promoter and AtAGPase downstream of the 35S or RbcS promoter were generated through Golden Gate assembly as described in 2.3.5 and contained a gene coding for hygromycin resistance (**Fig. 4.2.D – E**). Successful cloning of these constructs was confirmed firstly by orange-white selection. The Level 2 vector used for the Golden Gate cloning, pAGM4723, contained a gene coding for

canthaxanthin synthesis, an orange carotenoid pigment. This sequence is excised during the cloning process. As such, when *E. coli* are transformed with the construct, white colonies contain the desired construct whereas orange colonies do not (Geddes *et al.*, 2019). Successful assembly was confirmed by sequencing (Eurofins, London, UK). A pictorial illustration of plasmid structures, both donated and cloned, can be seen in **Fig. 4.2**. Plasmid maps generated by Geneious are available in **Supplemental Figures 1 – 5**.

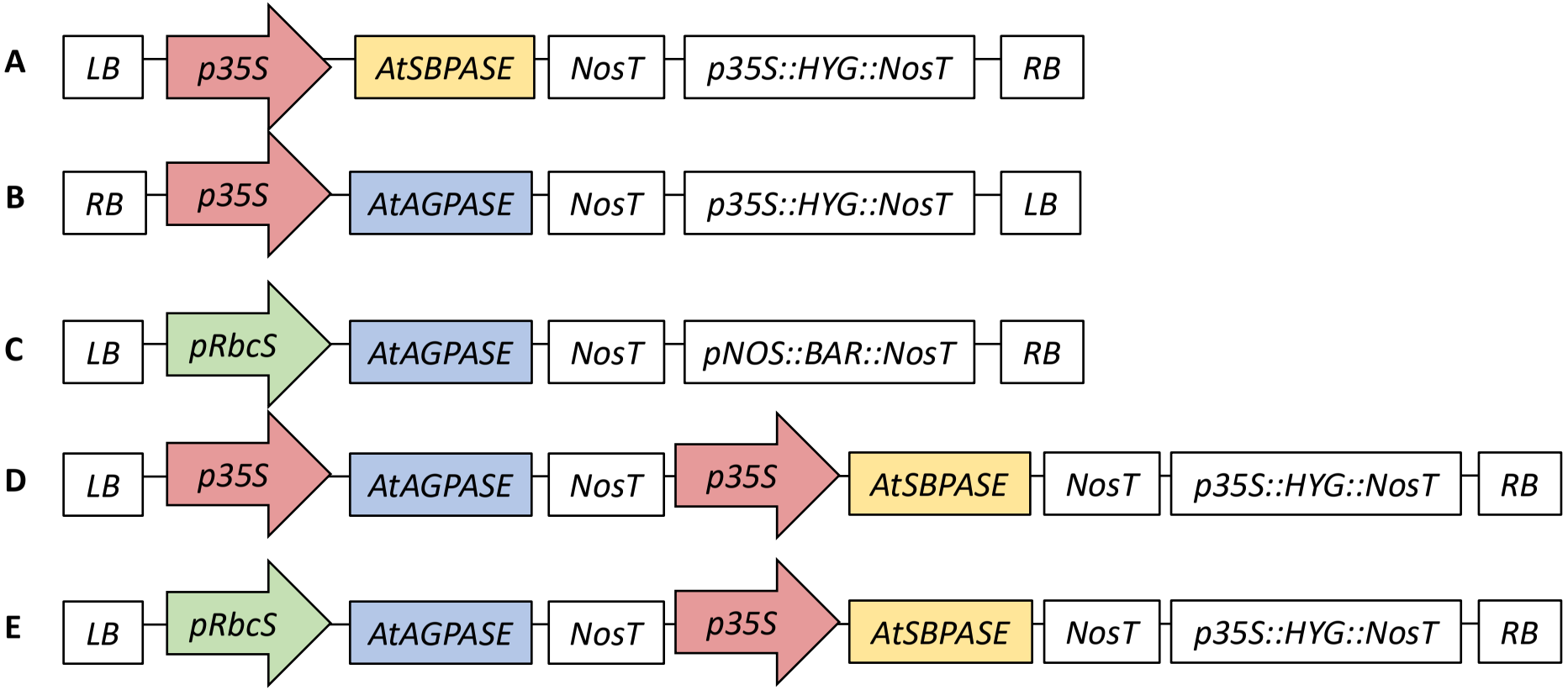

Fig. 4.2 Plasmid maps of constructs generated by Golden Gate Assembly containing the genes of interest. LB = Left Border; RB = Right Border; p35S = constitutive promoter from cauliflower mosaic virus; pRbcS = green tissue specific promoter from *Arabidopsis thaliana* RuBisCO small subunit; AtSBPASE = *Arabidopsis thaliana* sedoheptulose-1,7-bisphosphatase gene; AtAGPASE = *Arabidopsis thaliana* adenosine diphosphate glucose pyrophosphorylase gene; NosT = nopaline synthase terminator; pNOS = nopaline synthase promoter; HYG = hygromycin resistance gene; BAR = glufosinate resistance gene. **A:** pL2B-BAR-p35S-AtSBPase-tNOS. **B:** pL2B-HYG-p35S-AtAGPase-tNOS. **C:** pL2B-BAR-pRbcS-AtAGPase-tNOS. **D:** pL2B-HYG-p35S-AtSBPase-p35S-AtAGPase-tNOS. **E:** pL2B-HYG-p35S-AtSBPase-pRbcS-AtAGPase-tNOS.

4.3.2 Transformation

4.3.2.1 Theoretical Background

Transformation of cultivated strawberry is typically performed on leaf tissue due to its abundance, however almost any part of the plant can be successfully regenerated and is therefore potentially suitable for transformation (Folta and Dhingra, 2006). Due to the relatively long time it takes to obtain strawberry seed, growing strawberry plants from seed is a less viable method of generating strawberry leaf tissue for transformation work. Instead, a method known as micropropagation is employed. First described for strawberry by Philippe Boxus in 1974 (Boxus, 1974), this involves the use of the hormones 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA), a cytokinin and an auxin respectively, to stimulate plant growth on an MS based agar substrate. Removal of most leaf and all root tissue and re-planting the crown tissue in media (known as "subbing") keeps the plant growing on fresh nutrients and carbon. The leaf and root tissue will naturally regenerate. Using this method, plants can be stored indefinitely in sterile conditions, with fresh leaf material available every four weeks for use in transformation work.

As previously alluded to, transformation from leaf material requires regeneration of plantlets from transformed callus tissue. While it is known that different varieties may require different auxins and cytokinins in different concentrations to regenerate (Jones *et al.*, 1988), work carried out in the model cultivar Calypso identified 1.0 mg L^{-1} thidiazuron (TDZ) and 0.2 mg L^{-1} 1-naphthaleneacetic acid (NAA) the most efficient combination for regeneration (Passey *et al.*, 2003). As such, this combination was used for regeneration in this methodology.

While micropropagation is excellent in a commercial setting, it is important to consider the effect this process has on the physiological characteristics on strawberry plants. Due to the presence of carbon in the growth substrate, plants in micropropagation have little to none photosynthetic capacity, however new leaves developed once transplanted to soil are able to

photosynthesise normally (Grout and Millam, 1985). Furthermore, chlorophyll fluorescence analysis has revealed that photosynthesis of these lines is no different from plants which haven't been micropropagated (Borkowska, 2001). This means that photosynthetic analysis isn't affected by the transformation process, improving the validity of this research. However, when analysing yield, it must be noted that excessive subbing of strawberry can lead to overflowering once transplanted to soil (Boxus, 1999). As such, if fruit yield is measured, control lines must also be micropropagated to ensure yield increases are derived from transformation and not from subbing alone.

A number of challenges exist when it comes to the transformation of cultivated strawberry. Some commercial lines and their progeny are fully recalcitrant to regeneration, such as the long-time market staple "Elsanta", and it has been demonstrated that this trait is genetically inherited (Passey *et al.*, 2003). Additionally, strawberry leaves contain a high concentration of phenolic compounds that can be toxic to transformed cells after the leaf has been cut (Palomo-Ríos *et al.*, 2018). Fortunately, dark incubation can reduce the degree of phenolic exudation from the leaf and can stimulate greater regeneration of cut tissue (Landi and Mezzetti, 2006).

4.3.2.2 Cultivars Calypso and EMR-773 have sufficient regeneration capacity for use in transformation of leaf tissue

As previously discussed, successful transformation can only occur with plant lines capable of regeneration. Two cultivars were tested for their viability and interest. Firstly, cultivar Calypso was selected for its previously demonstrated high transformation rate and successful capacity for regeneration (see Wilson *et al.*, 2019 for example). The flaw of using such a cultivar is its lack of current relevance to industry, as it was phased out as a commercial variety in the 1990s due to crown rot susceptibility. Additionally, use of non-elite lines in photosynthesis experiments is often critiqued due to the effects of improved photosynthesis not always translating into the highest yielding crops (meaning it is often seen that these highest yielding crops still yield more than non-elite lines with improved photosynthetic traits). (Theeuwen *et al.*, 2022). Despite this, Calypso still made a good cultivar to test due to prior demonstration of its effective transformation. The second cultivar tested, EMR 773, was selected due to its exceptionally high yield in experimental breeding trials. Although now phased out (again due to crown rot susceptibility), this would be an interesting cultivar to test due to its exceptional yield characteristics, since aiming to increase the yield of an already high yielding line could unveil greater insights into photosynthetic limitations on strawberry fruit yield.

Regeneration of leaflet explants of Calypso and EMR 773 cultivars was tested through growth on shoot regeneration medium (SRM) without selectable markers over 12 weeks (see **Fig. 4.3**). Four week old leaf explants were subbed onto plates (**Fig. 4.3.A**) and subbed every four weeks onto fresh media to ensure a stable supply of hormones and nutrients was provided. Callus induction was visible after 2 weeks on medium (not shown) with differentiated leaf and root tissue visible after 12 weeks of growth (**Fig. 4.3.C**). Callus was divided to separate developing plantlets and moved to shoot propagation medium (SPM, **Fig. 4.3.D**). Phenotypically normal leaf and root development was visible 2 weeks after division and transfer to SPM. This

work confirmed the suitability of Calypso and EMR 773 for transformation experiments and that the media used was sufficient for rapid regeneration of both cultivars.

A third experimental line, ever-bearing variety EMR 2434, was also tested this way. EMR 2434 was an interesting selection due to its exceptionally high yield but poor fruit sugar content. Regeneration of this variety was unsuccessful however, with regeneration progressing only to the callus stage. This may be explained by its genetic heritage. As previously noted, regeneration of different strawberry lines varies considerably on a cultivar to cultivar basis (Passey *et al.*, 2003) and EMR 2434 may have inherited genes from a lineage recalcitrant to regeneration. It may also have failed to regenerate due to the concentrations and types of auxins and cytokinins in the regeneration media, since the regeneration efficiency of different cultivars is also highly sensitive to this (Passey *et al.*, 2003).

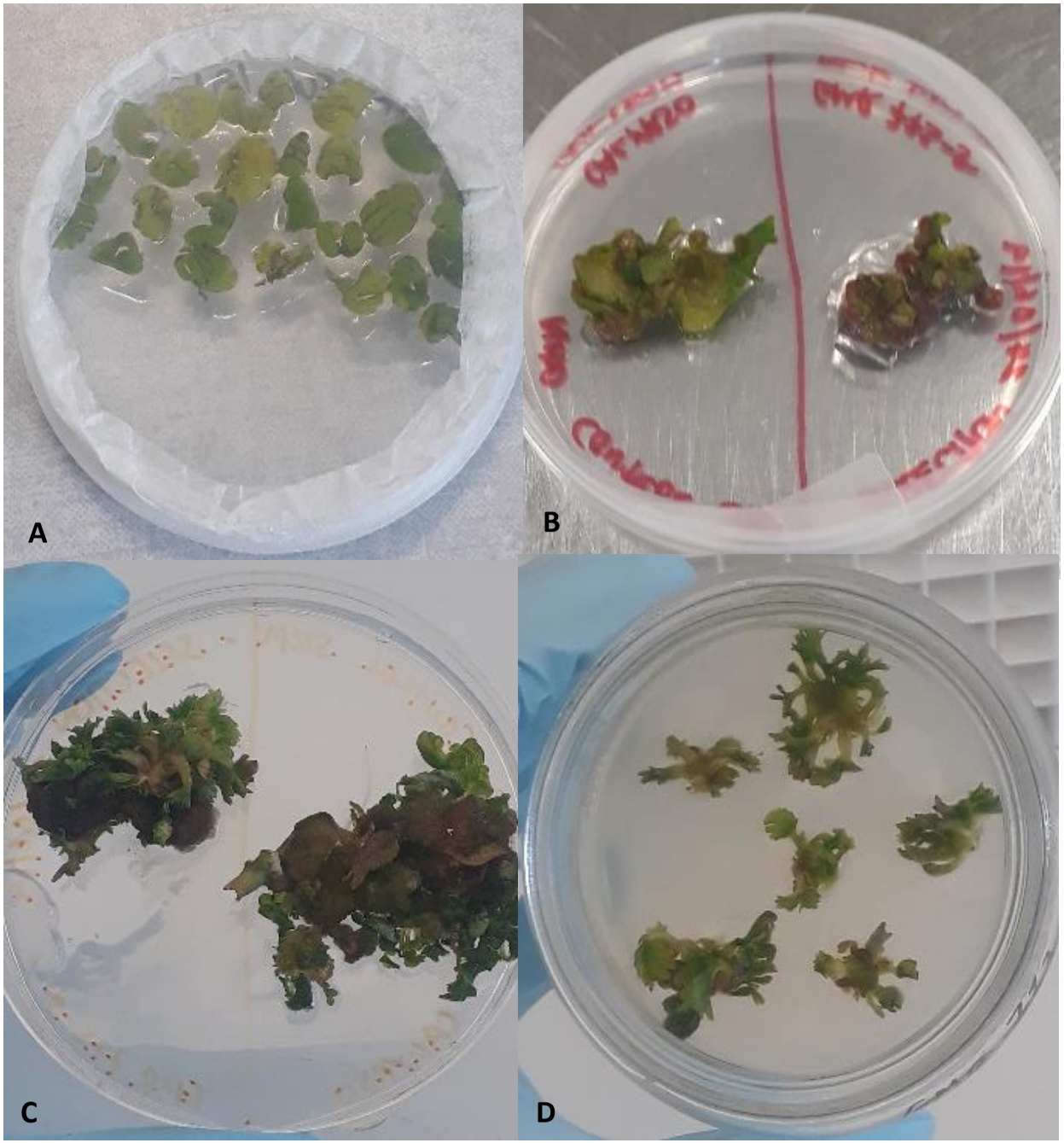

Fig. 4.3: Timeline of strawberry regeneration from leaflet explants to separated strawberry shoots. **A:** Scored leaflet explants from 4 week old strawberry plants (Calypso cultivar shown) on shoot regeneration medium (SRM). **B:** Explants at 4 weeks of growth on SRM (Calypso left, EMR 773 right). **C:** Explants at 12 weeks of growth on SRM (Calypso right, EMR 773 left). **D:** Separated regenerated strawberry shoots (EMR 773 cultivar shown) on shoot propagation medium (SPM).

4.3.2.3 Type and concentration of selectable marker markedly affects regeneration and organogenesis of transformed explants

Leaflets were transformed as described in 2.4.3 and placed onto SRM containing the appropriate selectable marker. These were glufosinate and hygromycin. Hygromycin was initially at a concentration of 50 mg L^{-1} and glufosinate at a concentration of 10 mg L^{-1}. These concentrations were set quite high to prevent the development of chimeric lines and had been described for successful selection in previous work (Wang *et al.*, 2004; Mathews, Wagoner, Kellogg, *et al.*, 1995). However no callus induction was visible at these concentrations, indicating that there was a flaw in the protocol.

To help prevent this inhibition of regeneration, hygromycin concentration was lowered to 25 mg L^{-1} and glufosinate to 5 and later 3 mg L^{-1}. To further aid transformation of leaf tissue, leaflet explants were not subbed into ticarcillin (TCA) containing media until after a week in the light. The longer exposure to agrobacterium was theorised to increase chance of successful infection of the leaf tissue. Despite these changes, for leaflets exposed to glufosinate no successful method was found to allow growth beyond the callus stage. It is apparent that glufosinate is highly inhibitory of regeneration on strawberry leaf tissue for the varieties tested. Previous work that aimed to optimise the use of glufosinate in strawberry transformation had a very low successful transformation rate at only 5 % (Zakaria *et al.*, 2014) so it is perhaps unsurprising that it didn't work during this experiment.

Conversely, more success was seen with leaflets grown on lower concentrations of hygromycin. Strawberry leaflets transformed with plasmid E were grown on hygromycin selection (See **Fig. 4.4**) and callus development observed eight weeks after transformation. Initial plantlet development was observed five months after transformation. This is much slower than was seen in control leaves, where plantlet development was sufficient for excision from the callus after 12 weeks. This demonstrates that hygromycin also has an inhibitory effect on

strawberry regeneration, however it is not sufficient to prevent regeneration from callus completely as seen in leaflets exposed to glufosinate. Plantlets developed on callus consistently at different rates. Large plantlets were excised from the callus and planted onto Fragaria rooting medium (FRAG-R) to encourage root induction. Fully rooted lines were then transferred to SPM for maintenance in tissue culture. In total, greater than 50 independent rooting strawberry lines expressing the double plasmid were generated.

The slow regeneration of strawberry explants may be due to the relatively high concentration of hygromycin used. One study found that even low levels of hygromycin (> 5 mg L^{-1}), glufosinate (> 0.1 mg L^{-1}) and kanamycin (> 12.5 mg L^{-1}) was sufficient to drastically inhibit regenerative rate of cultivar "Strawberry Festival" (Folta *et al.*, 2006). Additionally, previous work has used concentrations as low as 10 mg L^{-1} of hygromycin for selection (Nyman and Wallin, 1992; Mathews, Wagoner, Kellogg, *et al.*, 1995), however successful regeneration was observed for concentrations as high as 50 mg L^{-1} (Mathews, Wagoner, Kellogg, *et al.*, 1995). This may explain the greatly retarded rate of regeneration observed in this work. An alternative hypothesis may relate to the age of the plants themselves. Stocks were donated by NIAB EMR and used throughout the experiment, leading to many subbing passes. It has previously been observed that excessive subbing can induce unusual traits, such as excessive flowering (Boxus, 1999), and it has been hypothesised that this could be caused by DNA methylation (Boxus *et al.*, 2000). Recent work has demonstrated a causal link between DNA methylation and the capacity for callus induction and organogenesis in strawberry (Liu *et al.*, 2022). While the authors were unable to fully elucidate the intricacies of this relationship, it is apparent that epigenetics plays a significant role in the capacity for successful regeneration of strawberry leaf. This may further explain the delayed regeneration observed in this experiment.

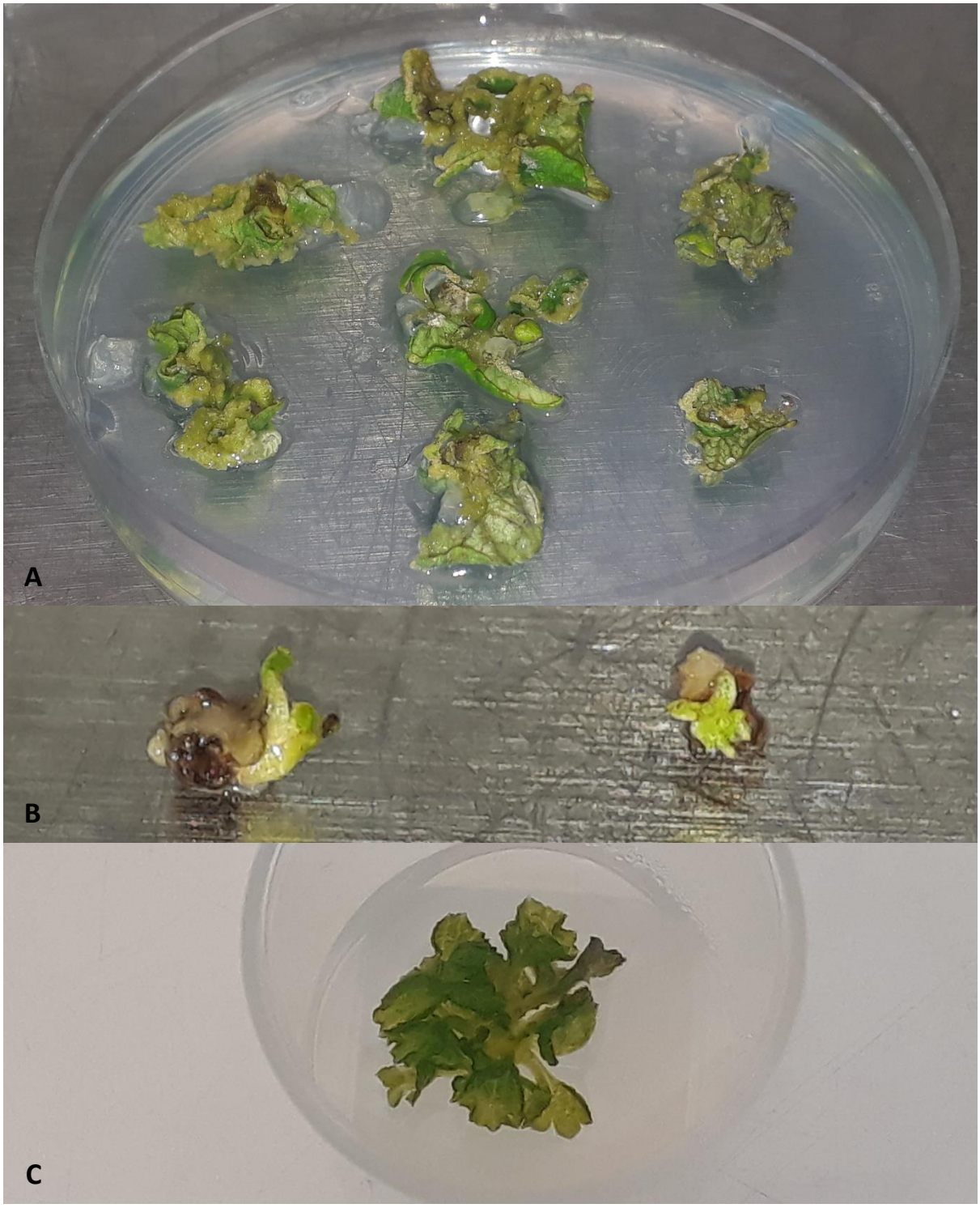

Fig. 4.4: Regeneration of strawberry leaf tissue on hygromycin selection. **A:** Callus induction after eight weeks on regeneration medium. **B:** Organogenesis at three months on regeneration medium. **C:** Advanced shoot organogenesis after six month months on regeneration medium. Shoot transferred to rooting medium.

4.3.2.4 Bacterial strain affects callus regeneration of two transformed strawberry cultivars

Further testing was also carried out exploring the effect of bacterial strain on the protocol's transformation efficiency. Two common strains of *Agrobacterium tumefaciens* were tested, namely AGL1 and EHA105. *Agrobacterium* is a bacteria with a naturally occurring capacity for gene transfection in plants, enabling the development of structures such as crown galls (Escobar and Dandekar, 2003). The discovery of this system led to the development of modified *Agrobacterium* strains which could be used to effect genetic modification, first successfully demonstrated in tobacco (Horsch *et al.*, 1984) and subsequently in tomato and petunia (Horsch *et al.*, 1985). Today, multiple strains of *Agrobacterium* exist that can be used for transformation methods, with AGL-1 and EHA105 two of the most commonly used (De Saeger *et al.*, 2021).

As demonstrated in **Fig. 4.5**, both tested strains were capable of successfully transforming leaflets, leading to visible callus at eight weeks of growth on hygromycin. While both strains tested were able to induce callus to at least a 40 % success rate, AGL1 was significantly more effective ($p < 0.05$) at inducing callus than EHA105 in both cultivars tested. Interestingly, EMR-773 exhibited a significantly greater capacity for callus induction than Calypso for strain EHA105 and a non-significant but still greater capacity for strain AGL1 (significant at the 10 % level). The effect of Agrobacterium strain on transformation efficiency of strawberry has been poorly studied (Palomo-Ríos *et al.*, 2018), though a number of strains have been previously tested and confirmed to be successful at transforming strawberry (although proportion of successful transformations has not been reported, full list described in Husaini *et al.*, 2011). Greater understanding of how bacterial strain affects the transformation process in strawberry could lead to more efficient, higher throughput transformation systems and therefore deserves further study.

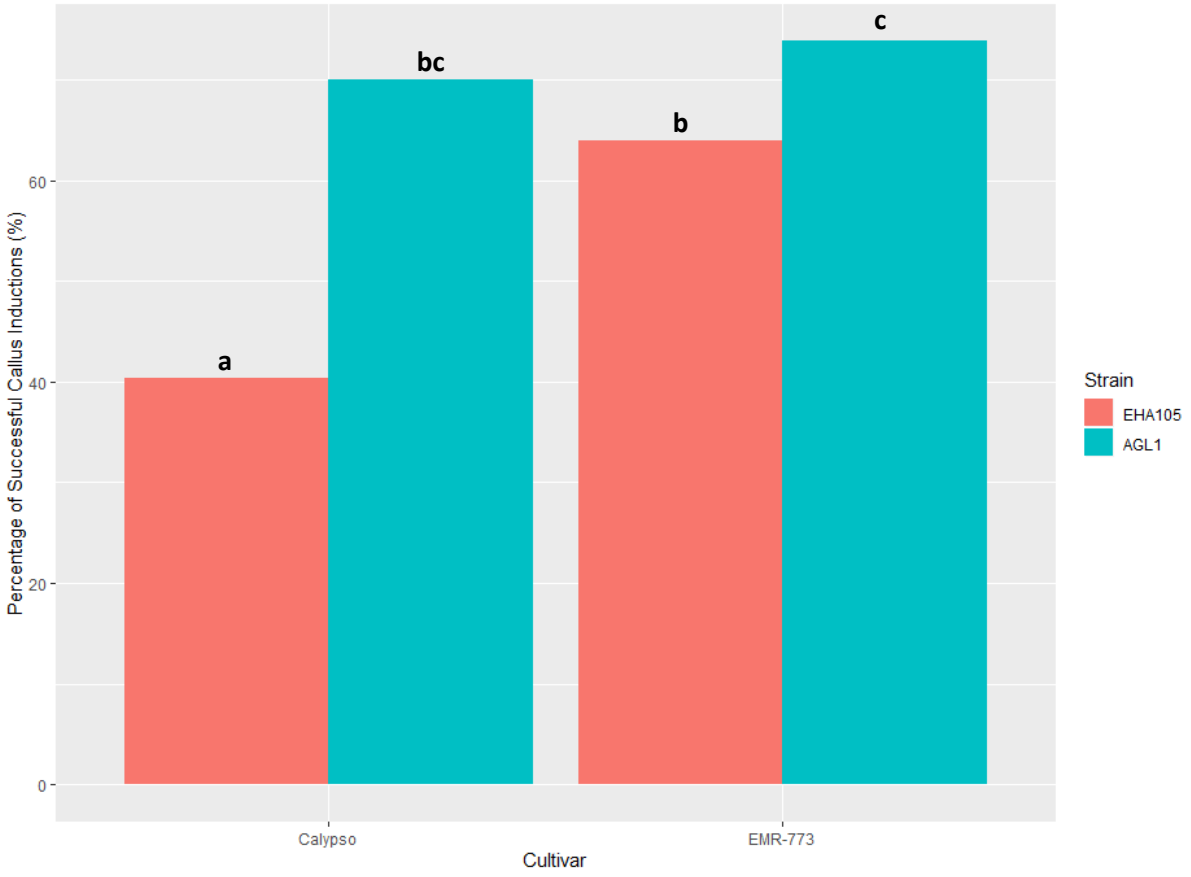

Fig. 4.5: Comparison of transformation efficiency of Agrobacterium strains EHA105 and AGL1 for strawberry cultivars Calypso and EMR-773. Letters indicate different significance for $p < 0.05$, tested via binomial test. n = 50 – 65.

4.3.2.5 Preparing transformants for analysis

After growth on FRAG-R for root induction, plants were subbed onto SPM for maintenance and SMM for bulking. Once sufficient numbers of plants had been generated of each line, plants were taken out of micropropagation and weaned onto medium grain vermiculite. Some plants were left in tissue culture as backups. Due to time constraints, a select number of lines with sufficient numbers of plants for both tissue culture backup and for the weaning process were taken forward in this process. These lines were numbered: 5, 6, 7, 8, 9, 13, 14, 20, 24, 45, 47 and the wild type (WT) control.

The weaning process is necessary to transfer between tissue culture and soil growth. This is due to micropropagation causing poor cuticle development, reduced leaf chlorophyll and photosynthetic characteristics, deformed stomata and poor root development (Mahendra *et al.*, 2020). After four weeks on vermiculite and increasing exposure, plants had developed much greater root structures and had new, better adapted leaves growing from the crown. Plants were potted in soil and moved to a growth chamber. See **Fig. 4.6** for images of this timeline.

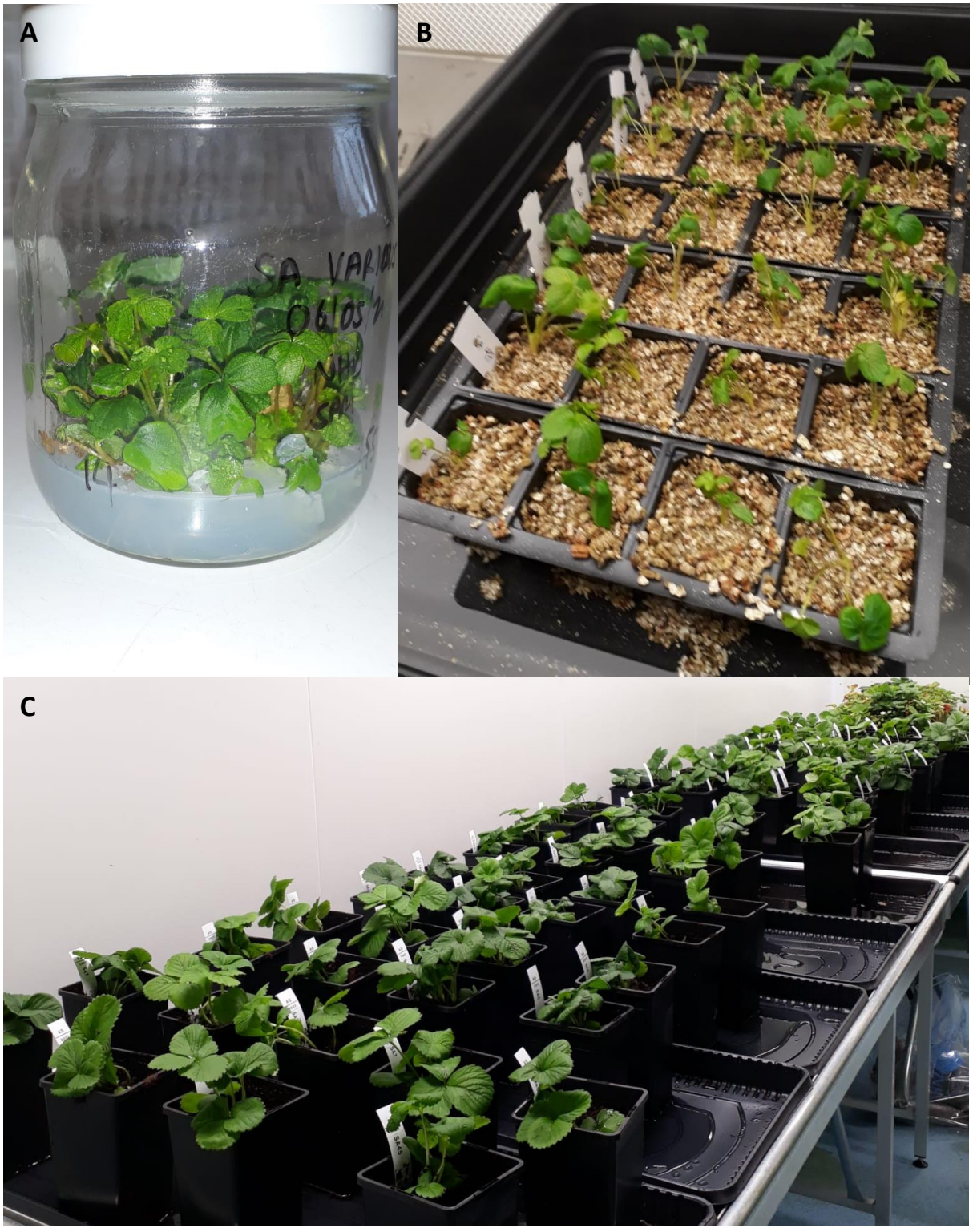

Fig. 4.6: Weaning of strawberry from tissue culture to soil. **A:** Fully regenerated and rooted transgenic plants, twelve months after initial transformation pass. **B:** Eight week old plantlets with minimum 10 cm roots planted in vermiculite. **C:** Plants four weeks after growth in soil (16 week old plants).

4.3.3 Confirming transgene expression

4.3.3.1. Testing of a phenol-free RNA extraction kit for RNA extraction of strawberry leaf tissue

Due to the high concentration of polyphenols and polysaccharides in strawberry leaves (Giampieri *et al.*, 2014), efficiency of RNA extraction is often compromised. Phenol-chloroform isolation of RNA from strawberry leaves has previously been shown to yield large quantities of high quality RNA, suitable for next-generation sequencing (Ishibashi *et al.*, 2019). However this does come with drawbacks. Due to the toxic nature of phenol, protocols which don't require the use of phenol are preferable. Previous work has identified 3 % cetrimonium bromide in the extraction buffer as a phenol-free solution for extracting high quality RNA from strawberry (Yu *et al.*, 2012). Furthermore, commercial kits have also been tested, such as the Qiagen Plant RNeasy Kit, and found to yield suitable concentrations and quality of RNA for RT-qPCR (Thompson *et al.*, 2003). This work tested the capabilities of the NucleoSpin RNA Plant and Fungi Kit (Macherey-Nagel) for extracting strawberry leaf RNA.

Extractions were tested on four week old and eight week old tissue of micropropagated plants with one whole tripartite leaf used for extraction. The kit was capable of extracting large amounts of high quality tissue in both four week old and eight week old leaves (see **Fig. 4.7**). The higher concentration of RNA seen in eight week old plants is reflective of the larger leaf size of the older material, but in both cases the amount of RNA extracted is much higher than required for RT-qPCR (5 ng mL^{-1}). This shows that one whole tripartite leaf is a larger amount of tissue than required for extraction and great enough quantities of RNA could likely be extracted from a single leaflet. It is interesting to note however that the 260/280 quality is significantly impaired by leaf age, reflecting increased DNA contamination in the extractions (see **Fig. 4.8**). This may be caused by increased polyphenol contamination of the sample. Leaf polyphenol content has previously been shown to increase with plant maturity in kale (Korus, 2011) and a

significant reduction in the 260/230 quality of the extracted RNA in eight week-old leaves versus four week-old leaves reflects this pattern in strawberry (although it is still in the acceptable range, > 2.0). Removal of DNA from RNA extractions is vital for RT-qPCR, since DNA present in the sample may skew the results through inducing greater fluorescence (Oñate-Sánchez and Vicente-Carbajosa, 2008). This identified four week old leaves as a suitable age for extracting strawberry leaf RNA. This is the same minimum age as plants propagated on runners need to be before severing from the parent to allow for sufficient root development, making this leaf age ideal for analysis. Overall the NucleoSpin RNA Plant and Fungi Kit is a suitable kit for strawberry RNA extraction and ideal for the work being carried out here.

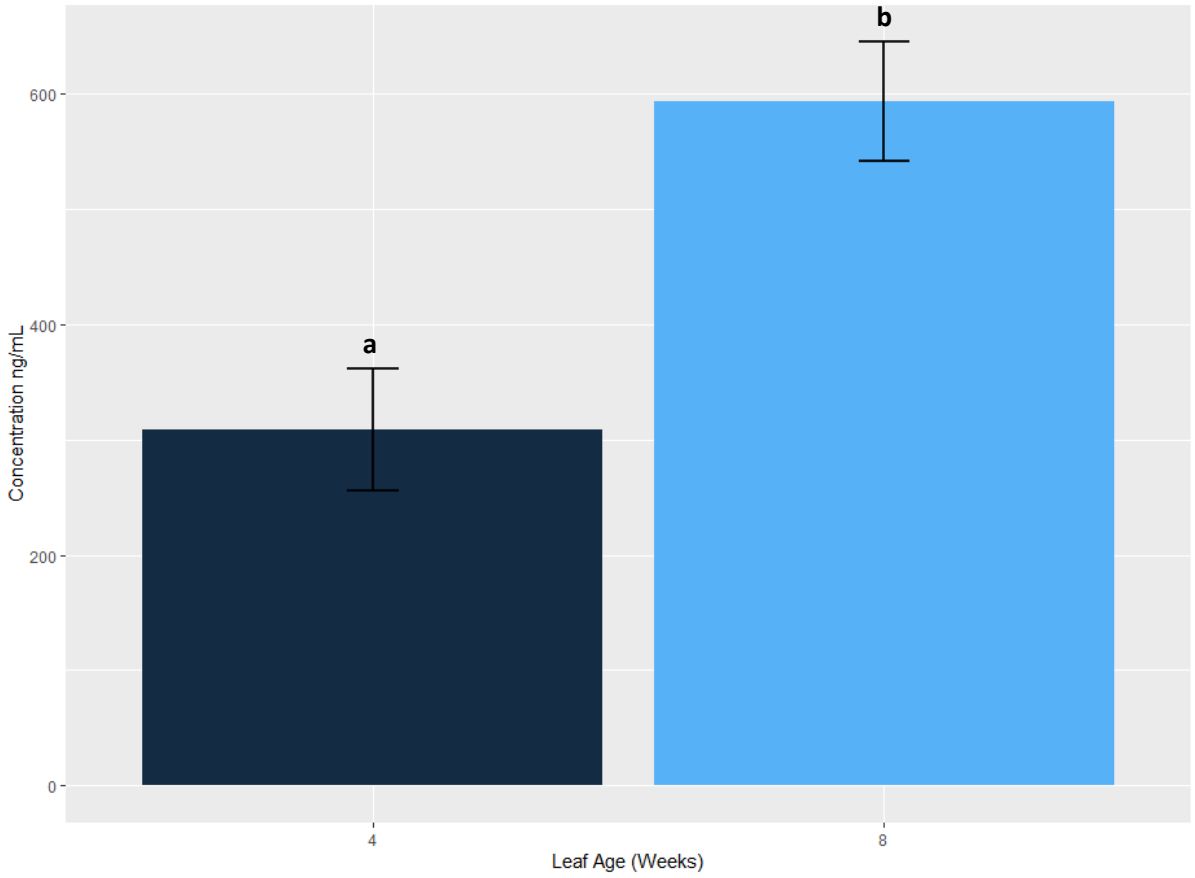

Fig. 4.7: Concentration of extracted RNA for leaf samples of two different ages. n = 4, error bars show standard deviation. Different letters indicate significantly different results (T-Test, $p < 0.05$).

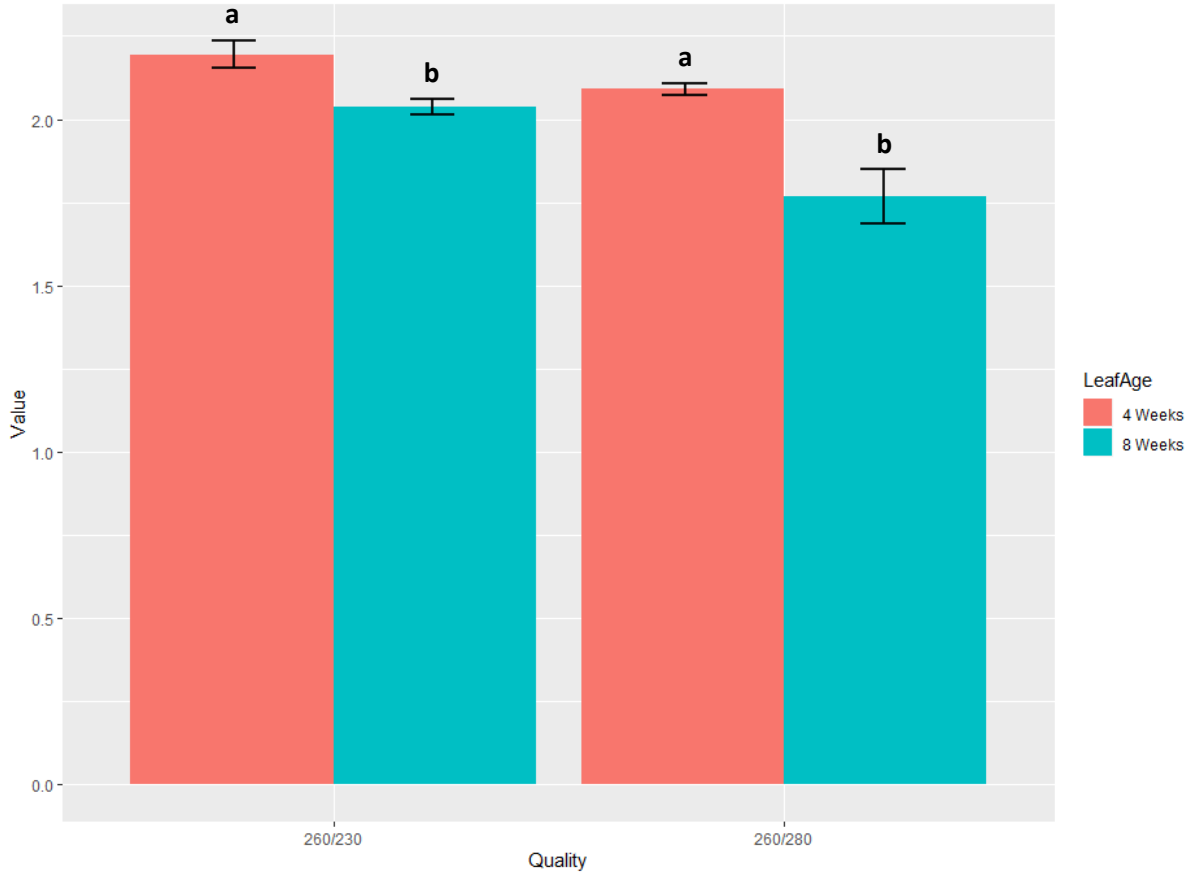

Fig. 4.8: Quality of extracted RNA for leaf samples of two different ages. 260/280 represents DNA contamination with ~2.0 ideal for pure RNA. 260/230 represents other molecular contaminants (e.g. phenolic compounds) with 2.0 – 2.2 ideal for pure samples. n = 4, error bars shows standard deviation. Different letters indicate significantly different results (T-Test, $p < 0.05$).

4.3.3.2. qPCR of transgenic lines confirms high expression of transgenes and reveals a line with minimal SBPase activity

To confirm expression levels of transformed genes, RNA was extracted from four week old plants grown in soil. This was done because in the soil, leaf photosynthetic enzymes are expressed normally unlike in tissue culture (Grout and Millam, 1985). If transgene silencing were occurring, this approach would ensure it would be identified through RT-qPCR. Extracted RNA was converted to cDNA via reverse transcription. The concentration and purity of these cDNAs is detailed in **Table 4.1**.

Primers for qPCR in strawberry must be designed to catch the full potential range of the strawberry genome. Cultivated strawberry is an octoploid with four subgenomes: *Fragaria vesca*, *Fragaria nipponica*, *Fragaria iinumae* , and *Fragaria viridis* (n = 7, 4 x 2n = 56) (Edger *et al.*, 2019). While the vesca subgenome is dominant for ~70 % of genes (Edger *et al.*, 2019), it cannot be assumed that this subgenome is dominant for any single gene. As such, primers for strawberry housekeeping genes were taken from papers that had identified these genes as good targets. These genes coded for 26srRNA (Lin *et al.*, 2021), glyceraldehyde 3-phosphate dehydrogenase (GAPDH1) (Clancy *et al.*, 2013) and endoplasmin-like protein (ENP1) (Clancy *et al.*, 2013). Additionally, primers were designed for strawberry SBPase and AGPase to explore transgene expression relative to the same genes in strawberry. Nucleotide BLAST searches on www.rosaceae.org were used to ensure primers aligned maximally to all genomes to try and achieve the fullest coverage. Unfortunately, a primer pair for SBPase could not be found. The AGPase primer was successful however and identified a > 85% increase in the degree of AGPase expression relative to host expression in all lines (see **Supplemental Table 10**). Primers for the transgenes and strawberry genes were designed amplify fragments of 194 – 201 bp in length to minimise error arising from amplification of different length fragments.

Results of the RT-qPCR can be seen in **Fig. 4.9**. Relative expression was calculated by subtracting the "cycles to threshold" (cT) value of the housekeeping gene from the cT value of the transgene. The cT value is the point at which well fluorescence increases beyond an arbitrarily set minimum threshold (Goni *et al.*, 2009).This can be used to confirm differences in gene expression of wells containing the same starting quantity of cDNA. Additionally a melt curve was applied at the end of the programme to check that only a specific fragment had been amplified (Ruijter *et al.*, 2019). A smooth baseline and a single peak was visible across all qPCRs carried out (apart from those looking at strawberry SBPase were no peak was visible), demonstrating the assay was specific to a single fragment.

The RT-qPCR identified two very important results. Firstly, the wild type line showed no expression of the transgene, confirming that the measured expression in the transgenic plants is derived solely from expression of the transgene and has not captured the expression of any strawberry genes. Secondly, expression of SBPase in line 45 wasn't detectable. These results are reflected in the large negative bars seen in **Fig. 4.9**. Expression of not every gene in transgenic plants transformed with a multigene plasmid is a known phenomenon and may be caused by transgene silencing, failure of the transgene to express sufficiently or failure of transgene insertion (Halpin, 2005); these results indicate that one or more of these has occurred here. From this point, this line was taken forward as singly overexpressing AGPase. Relative expression of all other lines varied within a ~2-fold range relative to housekeeping genes and expression of both AGPase and SBPase was detected. This gave rise to three lines, termed "WT", "A" and "SA" for "Wild Type", "AGPase overexpressing" and "SBPase-AGPase co-overexpressing", to be taken forward for phenotyping. SA lines 9, 13, 14 and 47 were selected for chlorophyll fluorescence analysis for having the most suitably sized, healthy daughter plants propagated on runners to be used as repeats alongside WT control lines and AGPase overexpressing line, 45.

This work would be better completed if complemented by determination of protein concentrations and enzyme activity, since expression of mRNA does not confirm if that mRNA has been translated, nor if any protein synthesised is truly active. This work was planned but not be completed due to unforeseen circumstances (see 6.3).

Line No.	cDNA Concentration (ng mL $^{-1}$)	260/280	260/230
5	368.8	1.90	2.29
6	282.6	2.13	2.24
7	366.7	1.77	1.79
8	352.0	1.73	2.31
9	376.3	1.82	1.81
13	299.2	1.93	1.60
14	407.4	1.68	1.73
15	426.2	1.62	2.28
20	236.3	2.11	1.57
24	276.4	1.71	1.50
45	296.0	1.65	1.86
47	225.8	1.84	1.93
WT	224.4	1.96	2.08

Table 4.1: Concentrations of cDNAs for different transgenic lines and wild type control (WT).

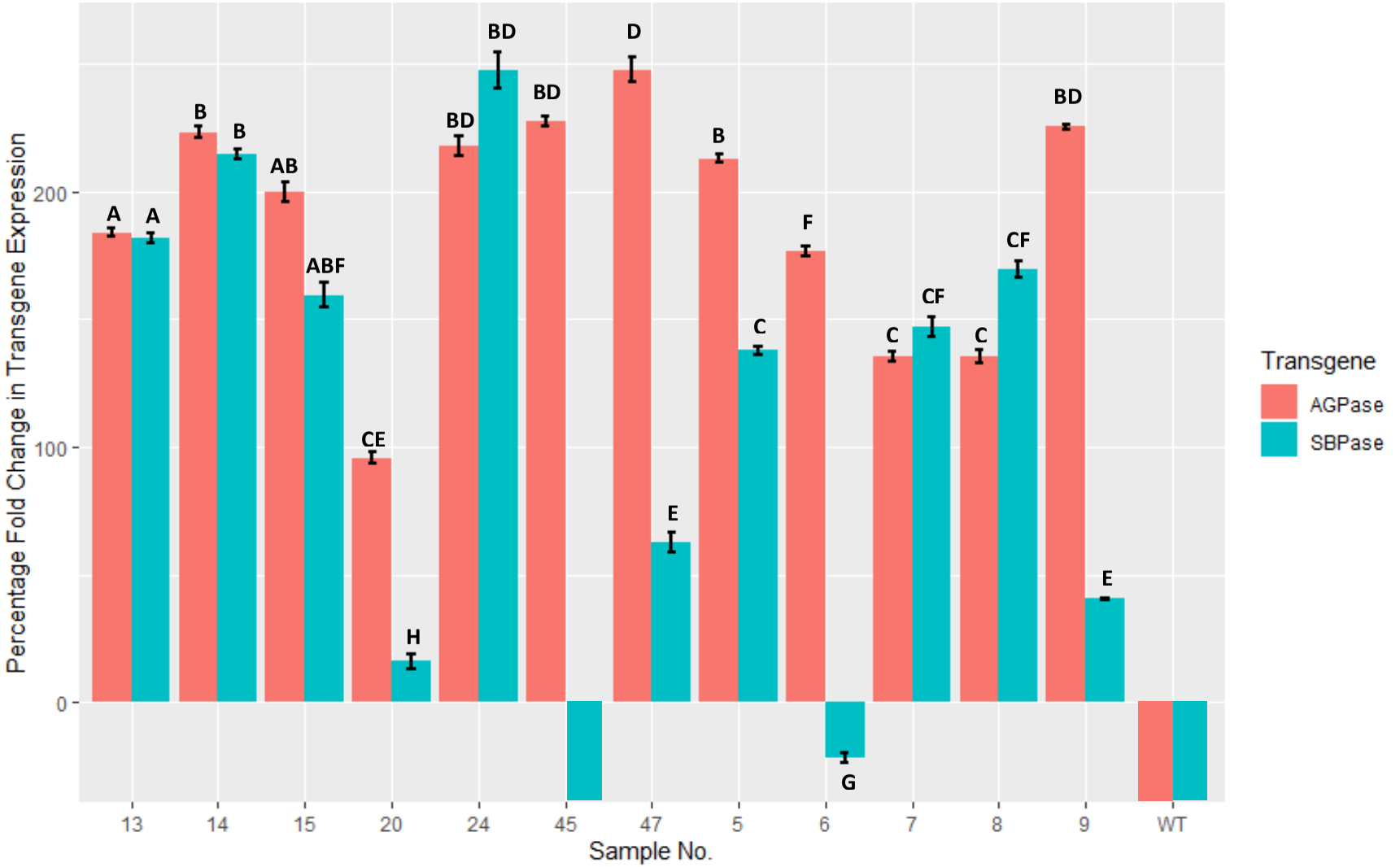

Fig. 4.9: RT-qPCR results showing fold-change in expression of *Arabidopsis* SBPase and AGPase relative to strawberry housekeeping genes 26srRNA, GAPDH1 and ENP1. n = 9 – 12, error bars show standard deviation. Where bars exceed axis limits, no transgene expression was detectable.

4.4 Summary

Strawberry plants overexpressing constitutive SBPase and green tissue specific AGPase were generated, with > 50 independent rooting lines to select from. Throughout this work, extensive challenges were faced that had to be overcome. The failure of the initial cloning process, due to kit faults, delayed plasmid construction and an excess concentration of the selectable marker hygromycin greatly delayed regeneration of shoots from the callus tissue. The cultivar Calypso has previously shown extremely high tolerance to the antibiotic kanamycin, able to regenerate in concentrations as high as 150 mg L^{-1} (Schaart *et al.*, 2004a), however it clearly does not display such high tolerance to hygromycin and very little tolerance for glufosinate. While this has reduced the scope of the transformation work achieved in this project, this lays the groundwork for future transformations using the cultivar Calypso in a hygromycin background and ultimately the process was successful in producing fully regenerated plants. The NucleoSpin Plant and Fungi RNA Kit (Macherey-Nagel) was identified as producing sufficient quality RNA for RT-qPCR to be carried out and RT-qPCR of transgenic versus wild type control leaves confirmed overexpression of SBPase and AGPase. One line, 45, showed minimal or null expression of SBPase and was defined as overexpressing only AGPase.

Future work in this area should continue to expand on understanding the interaction between agrobacterial strain and transformation efficiency. The significantly greater rate of callus induction observed when carrying out transformations using the AGL1 strain versus the EHA105 strain demonstrates that *Agrobacterium*-mediated transformation of strawberry has an as yet unexplored factor to be optimised. This may even aid the regeneration of cultivars with previously identified minimal capacity for regenerating tissue (e.g. Elsanta) (Passey *et al.*, 2003) and should be tested using a range of genetically variable strains and cultivars.

To conclude:

- Constructs were successfully transformed into Agrobacterium and from there into strawberry leaf material.
- Callus generated from the transformation successfully differentiated into fully developed plants, however this was delayed significantly by the choice of selectable marker and only worked for the double construct.
- Expression analysis revealed successful expression of both transgenes in all lines except the WT and one line (45) which had no detectable expression of SBPase, providing a line singly expressing AGPase.

Chapter 5 – Overexpression of SBPase and AGPase improves photosynthetic characteristics of cultivated strawberry individually and in concert

5.1 Introduction

The use of SBPase and AGPase overexpression to increase photosynthetic capacity of various model and crop species has been well studied (see 1.2.2 and 4.3.1.1 for further detail). However, there are multiple methods through which to study these effects. One such method is chlorophyll fluorescence. Chlorophyll fluorescence is a non-invasive measurement of photosystem II (PSII) activity (see **Fig. 1.2**) performed by measuring how green tissue responds to light pulses. Light harvested by chlorophyll can be used in photochemistry, including both photochemical and non-photochemical quenching (NPQ), or re-emitted as heat or light energy (Murchie and Lawson, 2013). These changes vary based on the state of the photosystem complexes. When light energy is harvested, it is transferred to the "special pair" of electrons at the PsbA/PsbD reaction centre (Gao *et al.*, 2018), exciting the electrons therein. While excited, the photosystem is described as being in the "closed" state, since any light energy harvested cannot be used to excite the electrons further. Once the electrons have transferred their energy to the photosystem they relax, entering the "open" state and able to be excited once more. Comparing how these changes in state affects light re-emission on exposure to a saturating pulse of light can be used to determine the efficiency of the photosystem complexes. At wavelengths below 700 nm, the contribution made by photosystem I (PSI) to these changes is minimal (Pfündel, 1998), allowing for reasonably accurate measurements of changes in fluorescence triggered exclusively by state changes of PSII. At room temperature, chlorophyll

fluorescence arises largely from light harvesting complexes of PSII rather than the reaction centre. Chlorophyll fluorescence is not the only method for studying photosynthesis. As carried out in Chapter 3, infra-red gas analysis (IRGA) can also be used to explore the CBC, determine J_{max} and V_{cmax} from A-Ci analysis to understand how photosynthesis is limited, and provide insights into secondary carbon metabolism and end-product limitation (which chlorophyll fluorescence is unable to accomplish). Ideally, both methods would have been applied to explore strawberry photosynthesis in this chapter but this was ultimately unable to happen (see 6.3).

Chlorophyll fluorescence is a well-studied technique in strawberry for measuring leaf photosynthetic capacity. Work has been carried out using this tool to understand strawberry photosynthetic responses to: drought and temperature (Razavi *et al.*, 2008; Na *et al.*, 2014; Zhou *et al.*, 2022; Choi *et al.*, 2016; Borkowska, 2002; Kadir, Sidhu, *et al.*, 2006; Archbold and Clements, 2002), UV-B radiation (Valkama *et al.*, 2003), salinity (Na *et al.*, 2014; Avestan *et al.*, 2021), sound (Meng *et al.*, 2012), humidity (Choi and Jeong, 2020), light quality (Choi *et al.*, 2016; Choi and Kang, 2019; Miranda and Williams, 2007), mycorrhizal inoculation (Borkowska, 2002), CO_2 fertilisation (Choi and Kang, 2019; Miranda and Williams, 2007; Keutgen *et al.*, 1997), pH (Malekzadeh Shamsabad *et al.*, 2021; Qi and Zhen, 2016) and nutrition (Osório *et al.*, 2014). Of particular note are those studies which exposed strawberry leaves to elevated atmospheric [CO_2] since this would stimulate photosynthesis and may reflect changes in genetically enhanced photosynthesis (see Chapter 1 for full review of this subject). Since this is, to the author's knowledge, the first time strawberry has been genetically modified to increase photosynthesis, this will also be the first reported instance of chlorophyll fluorescence being used to study the effects of genetically enhanced photosynthesis in strawberry. For a list of chlorophyll fluorescence parameters to be studied, see **Table 5.1**.

The hypotheses to be considered in this chapter are as follows:

- All lines will display equal maximum operating efficiency of PSII.
- Transgenic lines will display improved operating PSII efficiency relative to the WT.
- Transgenic lines will display elevated rates of NPQ relative to the WT.

Parameter	Calculation	Definition
F_O	Measured	Baseline fluorescence of dark adapted leaves with all reaction centres open
F_m	Measured	Total fluorescence after saturating light pulse for dark adapted plants
F’	Measured	Baseline fluorescence of the system under actinic light
F_m’	Measured	Total fluorescence after saturating light pulse for plants exposed to actinic light
F_v	$F_m - F_O$	Change in fluorescence caused by closing of all reaction centres in dark adapted plants

F_q'	$F_m' - F'$	Change in fluorescence caused by closing of reaction centres in plants exposed to actinic light
F_O'	Can be measured. Calculated as: $F_O/[(F_v/F_m) + F_o/F_m']$	Baseline fluorescence of light adapted leaves with all reaction centres open
F_v'	$F_m' - F_O'$	Change in fluorescence caused by closing of all reaction centres in light adapted plants
F_v/F_m	N/A	Maximum operating efficiency of PSII
F_v'/F_m'	N/A	Maximum operating efficiency of PSII efficiency in the light with all reaction centres open
ΦPSII	F_q'/F_m'	Actual operating efficiency of PSII in the light
qP	F_q'/F_v'	Non-linear estimate of open reaction centres, relates actual and maximum PSII operating efficiencies
NPQ	$(F_m - F_m')/F_m'$	Non-photochemical quenching

Table 5.1: Description of key parameters measured in chlorophyll fluorescence imaging and how they are calculated. Where no calculation is necessary, since parameter is recorded directly, parameter is described as "measured".

5.2 Materials and Methods

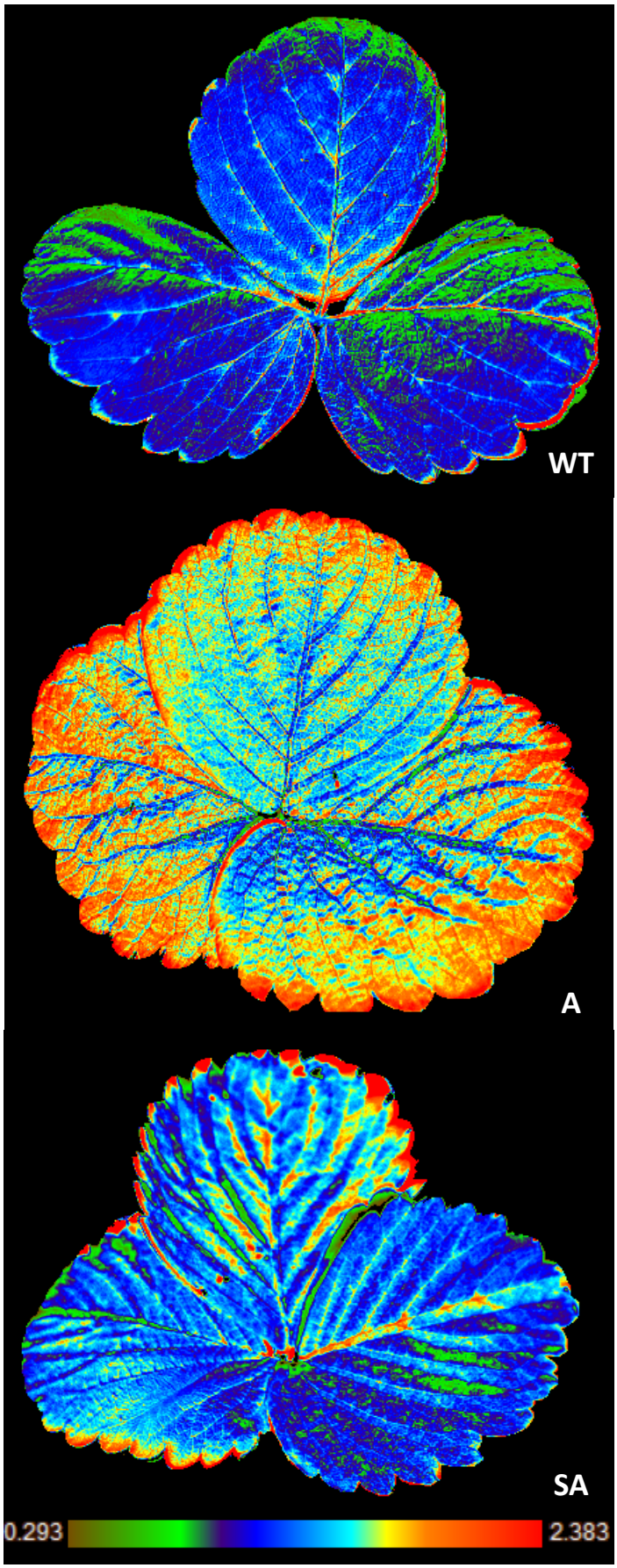

Fig. 5.1: Chlorophyll fluorescence images showing how full tripartite leaves were measured. Plants were placed 14 – 15 cm from the camera and strays deleted. Measurement shown here displays representative NPQ of wild type (WT), AGPase overexpressing (A) and SBPase and AGPase overexpressing (SA) plants exposed to 800 µmol m^{-2} s^{-1} actinic light. Hotter colours indicate greater NPQ as described in the colour gradient bar at the base of the image. See 5.3.2.4 for more details of effects of transformations on strawberry leaf NPQ.

Materials and methods were carried out as described in Chapter 2, section 2.5. On graphs throughout, different letters represent difference in significance $p < 0.05$. For example setup of chlorophyll fluorescence imaging, see **Fig. 5.1**.

5.3 Results and Discussion

5.3.1 Chlorophyll fluorescence phenotyping of transgenic lines

5.3.1.1. Control and transgenic strawberry leaves have consistent maximum quantum yield of PSII

To begin chlorophyll fluorescence experiments, four week-old plants were first dark adapted for 30 minutes and then exposed to a saturating pulse of light. It has previously been shown that older leaves (> seven weeks old) exhibit a deterioration in chlorophyll fluorescence parameters (Keutgen *et al.*, 1997), making this four week-old leaf age suitable for analysis. Dark adaption leads to all PSII reaction centres being open, since in the dark no photosystem chemistry can occur, and NPQ relaxes back to 0. Fluorescence of the leaf at this stage is measured by a low intensity light, sufficient to measure fluorescence but insufficient to stimulate the photosystem. This measure is termed F_0. The application of a saturating light pulse then closes all of the reaction centres. Since no photosystem chemistry was previously occurring, no light energy could be diverted via NPQ. Therefore, changes in measured fluorescence are due entirely to the closing of PSII reaction centres. This measure is termed F_m. Subtracting F_0 from F_m gives the metric F_v. Dividing F_v by F_m gives a metric of maximum possible efficiency of PSII, since it compares the change in fluorescence caused by opening all reaction centres (i.e. F_v) to the total measured fluorescence of the leaf with all reaction centres open (i.e. F_m) while no NPQ is occurring to divert light energy away from electron transport.

For accurate chlorophyll fluorescence measurements of NPQ, F_v/F_m must be ~0.83 (Björkman and Demmig, 1987). Previous work has demonstrated this is the maximum operating efficiency of PSII across a range of plant species. Deviation from this standard may indicate NPQ is occurring (Murchie and Lawson, 2013), a fault in the protocol or a fault in the machine. All strawberry leaves tested had an F_v/F_m of ~0.83 (see **Fig. 5.2**) and were therefore in the expected range. F_v/F_m is known to decrease with stress in strawberry (Na *et al.*, 2014), suggesting that these plants are fit for study. Furthermore, it has previously been shown that F_v/F_m is not correlated with strawberry fruit yield which has been linked to increased strawberry leaf photosynthetic rate (under unstressed conditions) (Choi *et al.*, 2016). It is therefore expected that no differences in F_v/F_m should be seen, regardless of differences in photosynthetic efficiency between lines.

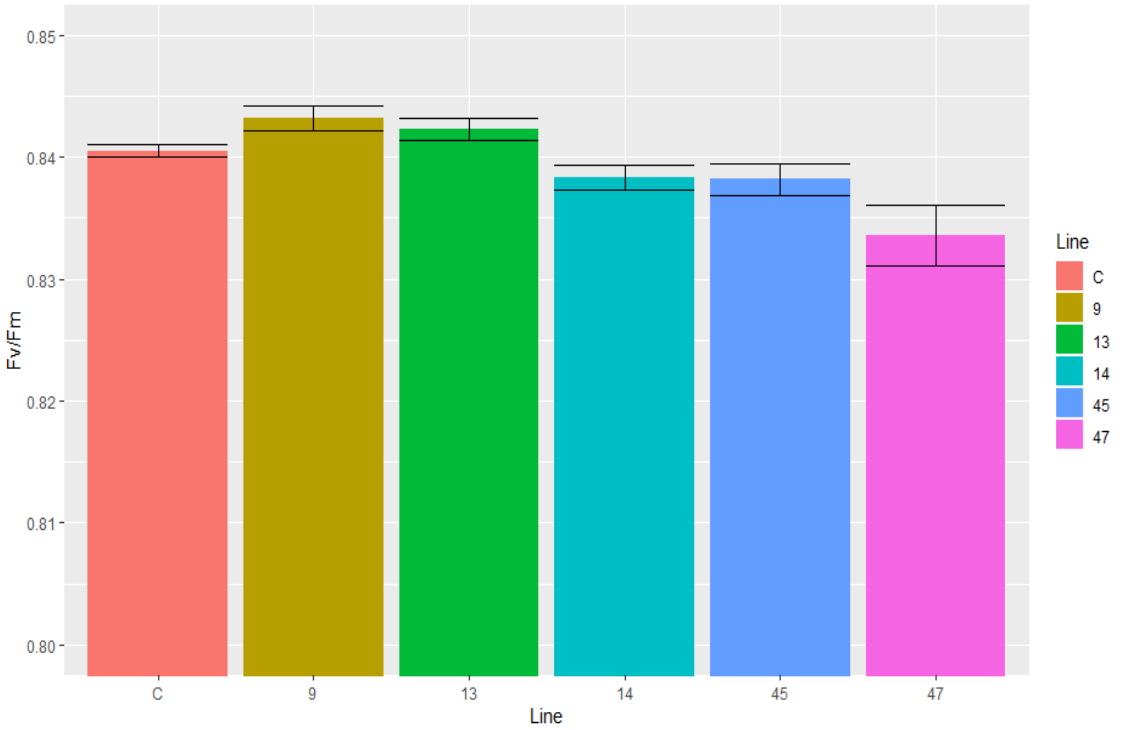

Fig. 5.2: F_v/F_m for six different lines. C = wild type control. 45 = AGPase overexpression only. All other lines co-overexpressing AGPase and SBPase. n = 5 – 6. Error bars show standard error. No significant differences between lines at $p < 0.05$.

5.3.1.2. Overexpression of AGPase alone is insufficient to increase PSII operating efficiency, however overexpression alongside SBPase leads to a non-significant increase

A different metric that can be studied in light adapted plants is F_q'/F_m', where F_q' represents the change in fluorescence when all reaction centres are opened and F_m' represents the total measured fluorescence from a saturating pulse while plants are still exposed to actinic light. The advantage of taking this measurement while the leaf is still exposed to the light is that the actual operating efficiency of PSII can be recorded, rather than a theoretical maximum. F_m' is measured directly. F_q' is calculated as follows:

$$F_q' = F_m' - F',$$

where F' represents the basal fluorescence of the leaf when exposed to light. Ensuring the F' has plateaued before taken measurements is necessary to demonstrate the leaf has fully adapted to the light.

Measurements were taken at two different light intensities, 400 µmol m^{-2} s^{-1} and 800 µmol m^{-2} s^{-1}. As seen in Chapter 3, differences in strawberry leaf photosynthetic efficiency begin to emerge around this higher light level, so to fully explore changes in phenotype associated with increased SBPase and AGPase overexpression, these two light levels were employed. It should be noted that, since these plants were grown in controlled conditions and plants in Chapter 3 were grown in the field, there may be some discrepancy between what light intensities are limiting on their photosynthesis.

At both light intensities, a small, non-significant increase in F_q'/F_m' was found in the double expressing line versus both the control and the AGPase only line, with this difference exacerbated at the higher light intensity (see **Fig. 5.3**). This suggests that AGPase is not rate-limiting on the efficiency of the photosystem while SBPase is.

While the exact, optimal balance of photosynthetic and related enzymes is unknown in cultivated strawberry, it has been theorized that an approximately 10-fold increase in AGPase activity relative to current activity *in planta* would be required to fully maximise the photosynthetic efficiency of higher plant photochemistry (Zhu *et al.*, 2007). However, overexpression of AGPase in green tissue has been shown previously to not affect photosynthetic carbon assimilation in tomato (Stark *et al.*, 1992). This work here agrees with this latter finding that AGPase alone does not influence the efficiency of photochemistry. It is however conceivable that this could still be rate-limiting on photosynthesis. Increased accumulation of starch has little negative feedback on photosynthesis (Petreikov *et al.*, 2009) and increased AGPase activity in the chloroplast would increase the strength of the transient starch pool, which acts as a sink in the chloroplast. Reduced sink capacity does induce negative feedback on photosynthesis and can limit photosynthesis even in favourable conditions (e.g. elevated [CO_2]) (Ainsworth and Bush, 2011). This suggests that overexpression of AGPase is required to increase photosynthetic efficiency by removing photosynthetic bottlenecks downstream of the CBB Cycle, so it may not affect photochemistry directly.

Conversely, overexpression of both SBPase and AGPase in concert led to a small, non-significant elevation of PSII operating efficiency (~2 %). This effect of SBPase overexpression has been observed before in a range of plants, including *Arabidopsis* (Andrew J. Simkin *et al.*, 2017), tobacco (Simkin *et al.*, 2015) and wheat (Driever *et al.*, 2017). In all these studies, increases in PSII photochemistry were small and insignificant but were correlated with increases in biomass and yield. This implies that even a small increase in PSII operating efficiency can effect a large change in plant physical characteristics. Additionally, as it has been shown that AGPase overexpression leads to little change in PSII operating efficiency, this change can be attributed largely to SBPase overexpression (although any enhancement effects caused by co-overexpression of the two enzymes cannot be determined). Despite these arguments however,

the increase remains non-significant. Whether a true enhancement of PSII operating efficiency is occurring remains unclear and warrants further study.

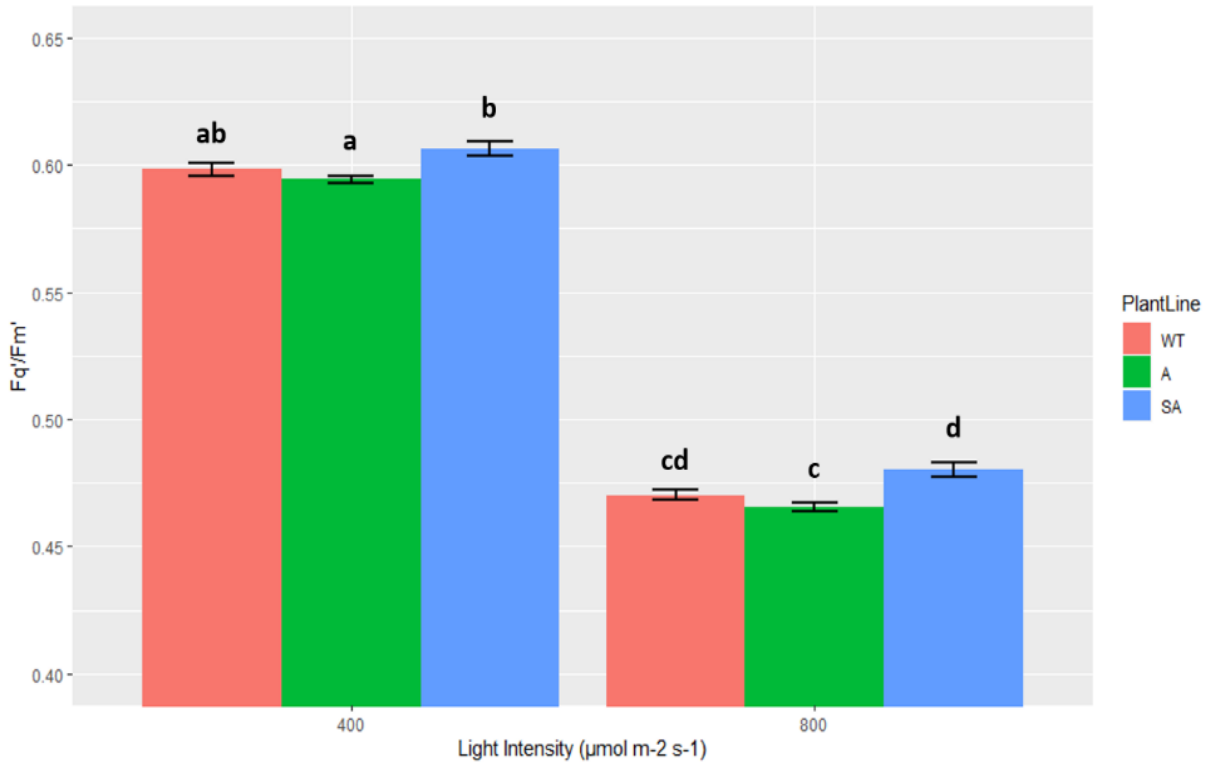

Fig. 5.3: F_q'/F_m' for three different lines at two different light intensities. WT = wild type control. A = AGPase overexpression only. SA = AGPase and SBPase co-overexpression. n = 6 – 12. Error bars show standard error. Different letters indicate significantly different results (T-Test, $p < 0.05$).

5.3.1.3. Transgenic lines show increased operating efficiency of PSII in the light relative to maximum operating efficiency due to a decrease in maximum operating efficiency

To unpick the differences in actual operating efficiency observed, two further metrics were taken. The term F_o' was calculated as follows:

$$F_o' = F_o/[(F_v/F_m) + F_o/F_m'],$$

as described in Oxborough and Baker, 1997. To measure F_o' experimentally, a low intensity, far-red pulse is applied a few seconds after actinic light is removed. In these few seconds, all reaction centres open since no light is available but NPQ continues as it takes longer to relax. This allows for measurement of the light adapted state with all reaction centres open, similarly to how F_o measures the dark adapted state with all reaction centres open. By using F_o', F_v' can be calculated as:

$$F_v' = F_m' - F_o',$$

where F_v' represents the change in fluorescence caused by the closing of reaction centres in a light adapted leaf. This gives rise to two different measurements. F_v'/F_m' can be used to determine the maximum operating efficiency of the leaf in the light adapted state, similarly to how F_v/F_m determines the same efficiency for the dark adapted state. F_q'/F_v' directly compares how fluorescence changes when the leaf is exposed to actinic light versus the maximum operating efficiency of the light adapted leaf; this measure is termed qP (photochemical quenching).

In both transgenic lines, an increase in qP was observed with this increase exacerbated by the higher light intensity (see **Fig. 5.4**). This can be interpreted as the transgenic plants having a greater fraction of open reaction centres and that the transgenic plants have greater actual operating efficiencies of PSII in the light relative to the maximum operating efficiency.

Additionally, qP is strongly correlated to increased fruit yield (Choi *et al.*, 2016), suggesting that the transgenic lines could yield greater quantities of fruits if measured.

This increase in qP is derived from a decrease is maximum operating efficiency of PSII in the light if all reaction centres were open (i.e. F_v'/F_m', see **Fig. 5.5**). A decrease in this theoretical maximum suggests an increase in NPQ (see 5.3.2.4). The most fascinating aspect of this however is the larger decrease seen in the line expressing AGPase alone versus the line overexpressing AGPase and SBPase in concert, indicating that NPQ must be greater in the line singly overexpressing AGPase. This is discussed in more detail below.

It is also worth noting that, despite having a greater fraction of open reaction centres, the operating efficiency of PSII of transgenic lines is still equal or greater than the wild type control. This implies that not only is a greater degree of photosynthesis being carried out in the co-overexpressing line (where PSII operating efficiency is increased) but that this operating efficiency could be further improved if more reaction centres closed. As previously observed in Chapter 3, strawberry leaves exposed to more light and with increased capacity for carbon assimilation did not fully maximise their photosynthesis to better conserve water. It is therefore not inconceivable that a similar pattern is being observed here. The potential for greater photosynthetic efficiency of transgenic strawberry leaves may be greater but other factors are limiting this. This is reflected in the need for fewer reaction centres to be closed to achieve sufficient electron transport for generating reducing factors for the CBB Cycle.

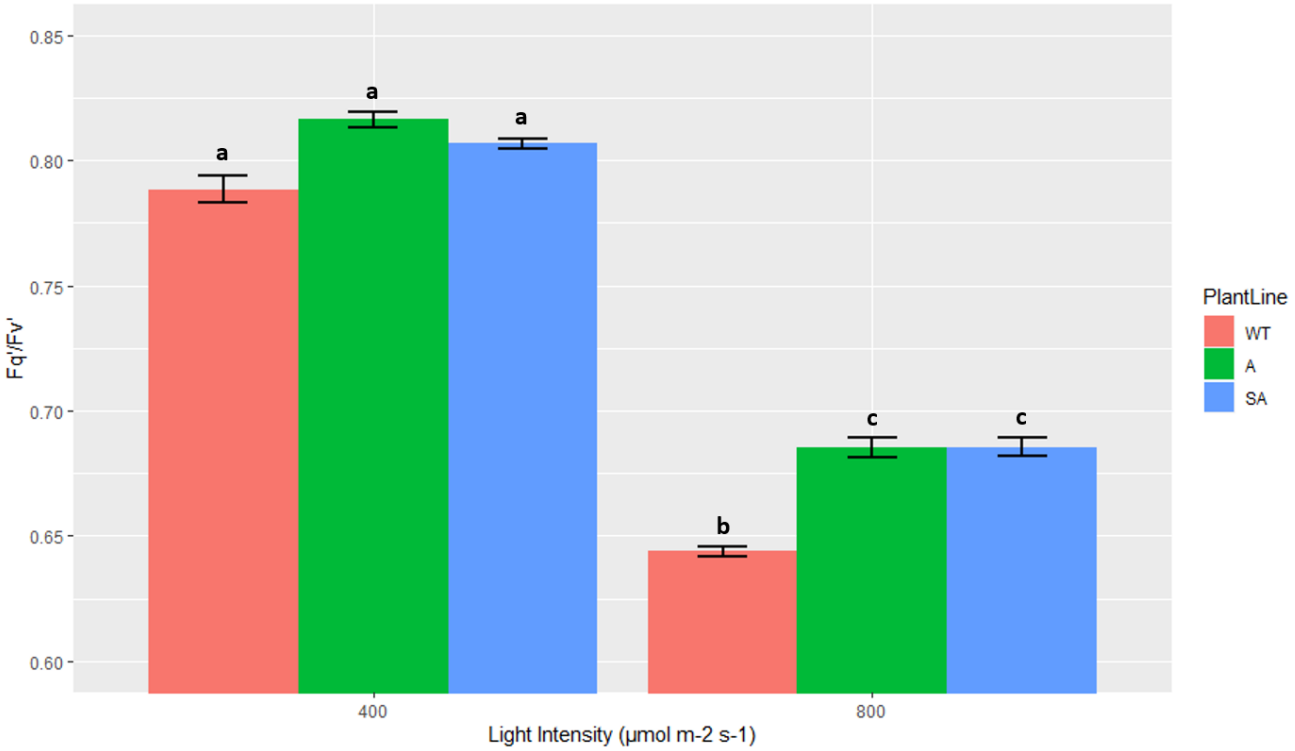

Fig. 5.4: qP for three different lines at two different light intensities. WT = wild type control. A = AGPase overexpression only. SA = AGPase and SBPase co-overexpression. n = 6 – 12. Error bars show standard error. Different letters indicate significantly different results (T-Test, $p < 0.05$).

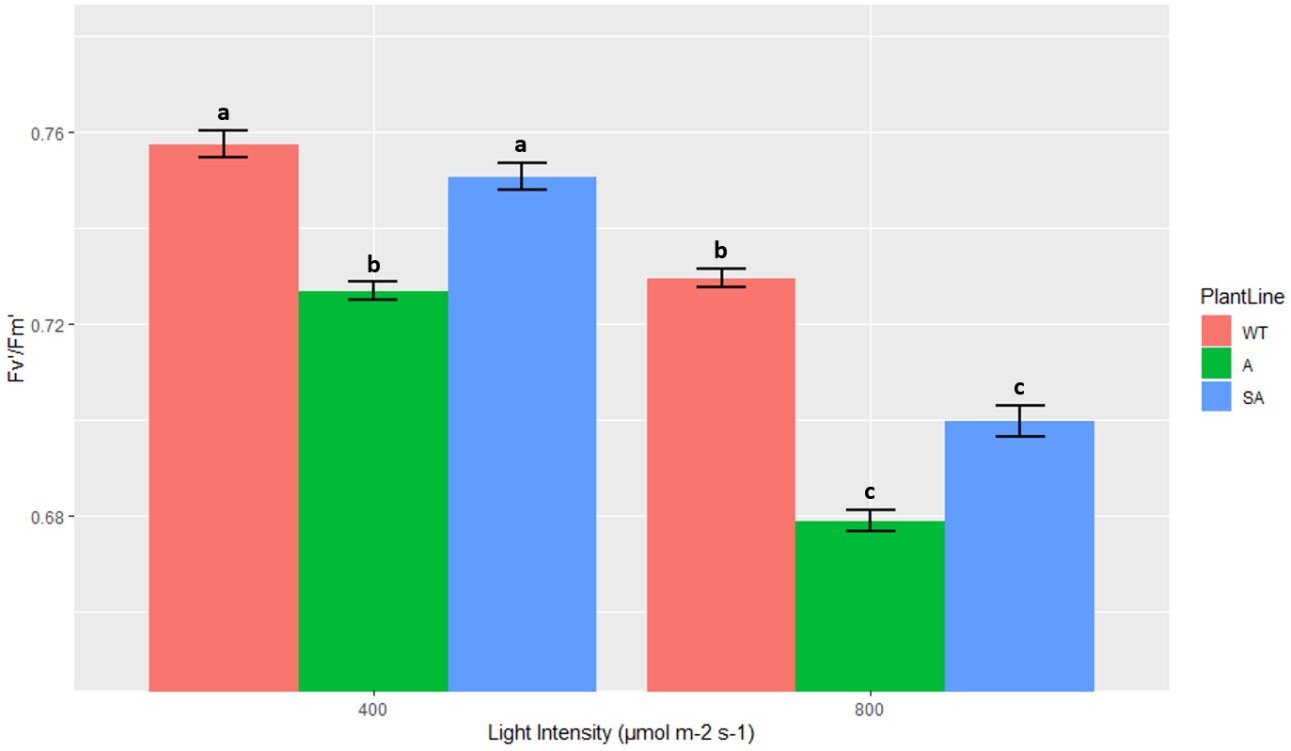

Fig. 5.5: F_v'/F_m' for three different lines at two different light intensities. WT = wild type control. A = AGPase overexpression only. SA = AGPase and SBPase co-overexpression. n = 6 – 12. Error bars show standard deviation. Different letters indicate significantly different results (T-Test, $p < 0.05$).

5.3.1.4. Non-photochemical quenching of the photosystem is greater in the transgenic lines especially at the more challenging light intensity

Non-photochemical quenching (NPQ) refers to light energy that is diverted away from the photosystem to prevent damage. Excessive light harvesting results in too much energy being absorbed by the photosystem. This energy is then processed in several ways such as use in the electron transport chain (i.e. photochemical quenching); re-emission as light (measured in chlorophyll fluorescence imaging) or heat (via NPQ); or the generation of radical oxygen species (ROS) (Khorobrykh *et al.*, 2020). ROS are formed when an electron's spin state is inverted, so two electron's with identical spin exist in the same orbital, or when oxygen is reduced (Khorobrykh *et al.*, 2020). This creates unstable, highly reactive free radicals that can react with the photosystem and trigger photoinhibition. This is mostly caused by damage to the protein PsbA (D1) (Li *et al.*, 2018), giving it a half-life of just 0.64 days (L., Li *et al.*, 2017). The mechanisms of NPQ exists to limit this damage. In the presence of light the xanthophyll cycle is activated, leading to the conversion of violaxanthin to zeaxanthin and of lutein epoxide to lutein (Jahns and Holzwarth, 2012). The structures of these two proteins enables increased dissipation of harvested light energy as heat. The reasons why this works exactly remain as yet unclear (Leuenberger *et al.*, 2017), though it is theorized that it is triggered by protonation of LHCII antenna components which enables clustering of NPQ-active protein complexes around LHCII (in particular, the PsbS site) for dissipation of energy, with larger complexes enabling greater NPQ to occur (Ruban, 2016). Increased capacity for faster activation and relaxation of the xanthophyll cycle (through overexpression of violaxanthin and zeaxanthin de-epoxidases and PsbS, a photosystem protein) has been shown to increase biomass of tobacco and soybean in the field (De Souza *et al.*, 2022; Kromdijk *et al.*, 2016).

An interesting observation is made here. Both transgenic lines had greater rates of NPQ than the WT plants with this difference again exacerbated at the higher light intensity (see **Fig.**

5.6). This result is consistent with previous, unpublished work examining the impact of overexpression of rate-limiting enzymes on photosystem chemistry. With greater capacity for photosynthesis, it is perhaps expected that NPQ would decrease since more light energy could be diverted towards the electron transport chain. This is observed in the increased F_q'/F_m' seen in the transgenic lines overexpressing SBPase and AGPase together. However the opposite is seen. Furthermore, an increase in NPQ is also observed in the line solely overexpressing AGPase and as predicted this increase is greater than in the co-overexpressing line. Despite this, it has previously been shown that increasing capacity for NPQ leads to increases in photosynthesis and yield (Hubbart *et al.*, 2018) further demonstrating the improved photosynthetic qualities of the transgenic lines. Additionally, NPQ is well correlated to increased yield of strawberry (Choi *et al.*, 2016), suggesting that even the line overexpressing AGPase alone could still see an increase in fruit yield.

A suitable explanation for this observation has yet to be discussed in literature which explores how to increase photosynthetic efficiency through genetic manipulation (such as those discussed in Simkin *et al.*, 2019. However this work may reveal fresh insights. As previously discussed, overexpression of AGPase does not increase PSII operating efficiency in the same way as co-overexpressing with SBPase does but the increase in NPQ is still seen. There is limited work exploring the interaction between AGPase and NPQ. One previous study in *Arabidopsis* has suggested that downregulation of AGPase reduced starch synthesis and had limited effects on changes in NPQ (Thormählen *et al.*, 2013), implying that AGPase is not important in affecting this mechanism. However an increase in sucrose synthesis was seen. This suggests that, due to attenuated expression of AGPase, photoassimilated carbon was re-directed to a different sink pool within the chloroplast. It could therefore be argued that overall sink strength was not altered, merely the strength of different sinks within the leaf changed. If no reduction in sink strength occurred, would this cause any feedback on photochemistry? This deserves greater exploration. What is apparent from this work, is that increasing sink strength enables much

greater NPQ to be carried out. A possible explanation for this is that, while increased starch synthesis does not directly downregulate photosynthetic carbon assimilation (Stark *et al.*, 1992), it upregulates NPQ to divert energy away from the photosystem. This would help prevent over-accumulation of photosynthetic end products. Of course, this would then be associated with a reduction in photosynthesis, since more harvested energy is being dissipated. While this explanation remains incomplete, it asks new questions about the interaction between the regulation of photosystem chemistry and the end products of the CBC.

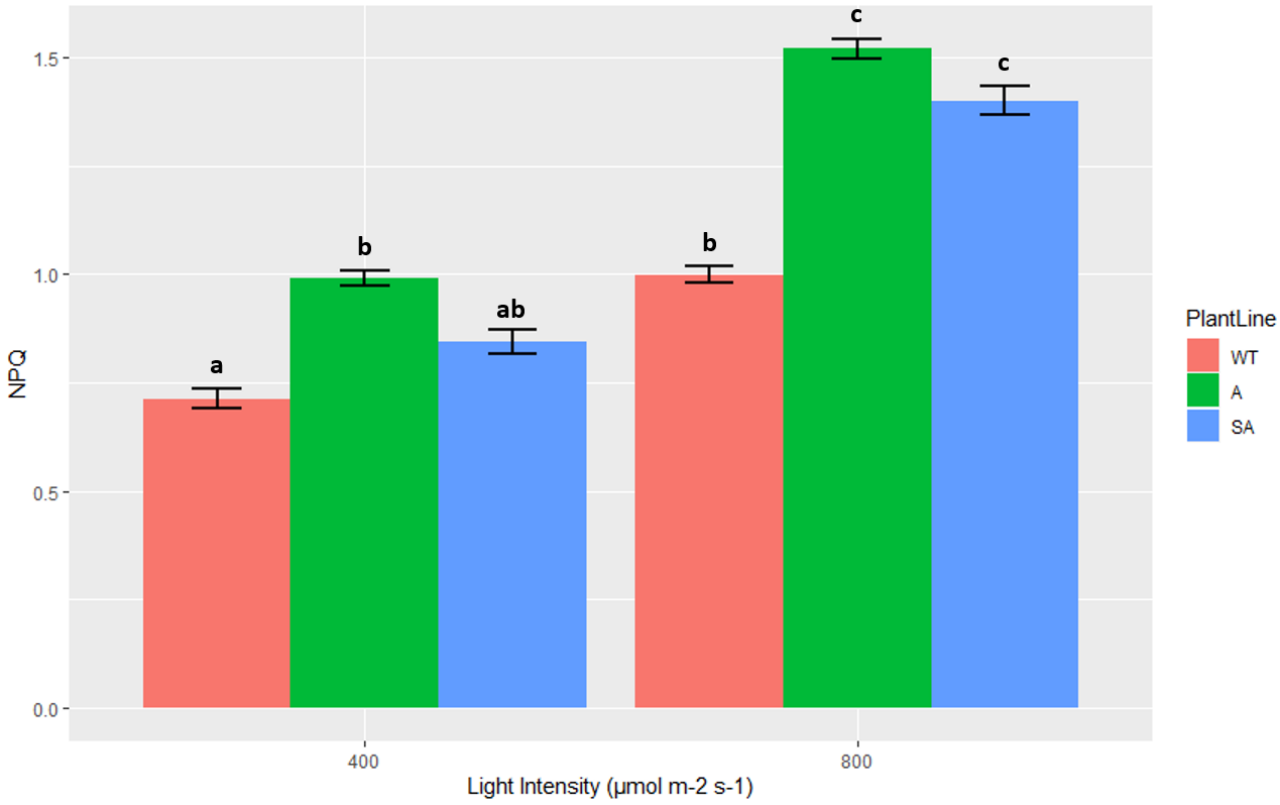

Fig. 5.6: NPQ for three different lines at two different light intensities. WT = wild type control. A = AGPase overexpression only. SA = AGPase and SBPase co-overexpression. n = 6 – 12. Error bars show standard deviation. Different letters indicate significantly different results (T-Test, $p < 0.05$).

5.4 Summary

This work has demonstrated that through overexpression of rate-limiting enzymes associated with photochemistry, the photosynthetic characteristics of strawberry leaves can be improved. The NucleoSpin Plant and Fungi RNA Kit (Macherey-Nagel) was identified as producing sufficient quality RNA for RT-qPCR to be carried out and RT-qPCR of transgenic versus wild type control leaves confirmed overexpression of SBPase and AGPase. One line, 45, showed minimal or null expression of SBPase and was defined as overexpressing only AGPase. To explore the phenotype of these overexpressers, four-week old strawberry plants propagated on runners were placed in a chlorophyll fluorescence imaging system. Co-overexpression of SBPase and AGPase was sufficient to non-significantly increase the operating efficiency of PSII and overexpression of either AGPase alone or in concert with SBPase led to increases in qP and NPQ that were exacerbated when exposed to a light intensity of 800 µmol m^{-2} s^{-1}. This is significant in the context of polytunnel grown plants since, at this light intensity and greater, leaves with greater capacity for photosynthesis perform photosynthesis more efficiently than their counterparts; this has concomitant effects on fruit yield (see Chapter 3).

Future work in this area needs to explore not only chlorophyll fluorescence of the photosystem but also leaf gas exchange to understand if there are differences in the quantity of assimilated carbon. This work was planned for this study but ultimately could not be carried out in time (see 6.3.3). Further to this, fruit yield and fruit flavour of these plants should be recorded. The correlation between increased photosynthesis and fruit yield has already been well established (see Chapter 3) and results from CO_2 fertilisation of strawberry implicate improved photosynthesis in enhanced accumulation of sugars, vitamin C and key flavour molecules (see **Supplemental Table 3**). Recording these data would neatly wrap up the work that has been begun here and provide even further insights into how strawberry photosynthesis can be genetically improved to increase yield and flavour.

To conclude:

- F_v/F_m was not significantly different between lines, indicating plants were healthy and unstressed.
- PSII operating efficiency was not significantly increased in transgenic lines, though a small, non-significant increase was observed in the double expressing line.
- NPQ was elevated significantly in transgenic lines grown at high light, indicating an increase in the activity of photoprotective mechanisms.

Chapter 6 – General Discussion

6.1 – Introduction

This chapter aims to bring together and further delve into the discussion given in the preceding chapters. The experiments carried out in this manuscript were not performed in isolation of one another and require comparison. This chapter also aims to evaluate the quality of the work carried out, determine flaws and obstacles of the methodology and provide insight into the future directions of both this project and the field as a whole.

Cultivated strawberry (*Fragaria x ananassa. Duch*) is a high-value berry crop representing an estimated £352.4 million of worth to the UK economy in 2021 (DEFRA, 2022). It is also a highly studied crop with a recent, high quality reference genome enabling much more advanced study at the molecular level (Edger *et al.*, 2019; Whitaker *et al.*, 2020). Further to this, transformation of cultivated strawberry is a well-established system with recent advances including the development of a CRISPR-Cas9 system, an especially remarkable feat considering all eight subgenomes must be targeted (Wilson *et al.*, 2019). It stands to reason then, that strawberry is an ideal candidate for exploring how genetic enhancement of photosynthesis may be employed in fruiting crops.

The efficiency of photosynthesis is a limiting factor on crop growth which is yet to be optimised (Long *et al.*, 2006). As such, many approaches with which genetic engineering may be used to enhance photosynthesis have been developed, such as the RIPE project (https://ripe.illinois.edu/) and the C4 rice project (https://c4rice.com/). These approaches are fully reviewed in Chapter 1. The work in this thesis builds on a workpackage developed by the RIPE project at the University of Essex exploring how multigene manipulation of rate-limiting

photosynthetic enzymes through gene stacking can be used to increase photosynthetic efficiency. This multigene approach has been applied with great success in tobacco (Simkin *et al.*, 2015; López-Calcagno *et al.*, 2020), *Arabidopsis* (Simkin *et al.*, 2017a) and rice (Gong *et al.*, 2015), leading to over 100 % increases in plant biomass in some instances. This thesis aimed to apply this method to strawberry. Understanding how fundamental biotechnology research in plant science can be applied to industrial settings is vital for plant science to achieve maximum impact; that is what this work tries to achieve. Greater production of strawberry fruits would not only increase profits for growers but also decrease costs for consumers, increasing the availability of healthier options. Better access to such products through economic growth is strongly correlated to reduced micronutrient malnutrition or "hidden hunger" (Gödecke *et al.*, 2018), so this work could give aid to the most disadvantaged.

In order for this to work, species-specific factors limiting photosynthesis in strawberry must be considered. As seen in previous studies, the same approach in one plant species is not always successful in another. For example, overexpression of transketolase in tobacco caused an auxotrophic phenotype (Khozaei *et al.*, 2015), whereas overexpression in rice had no measurable effects on phenotype (Suzuki *et al.*, 2017). Alongside genetic improvement of strawberry then, species-specific factors affecting strawberry photosynthesis were unpicked by exploring how photosynthesis varies with polytunnel row position. Historical data revealed that in polytunnels built on a north-south axis, plants grown in the centre rows, in particular the western central row (Row 4), had 10 % greater yield than external rows. This was attributed to increased photosynthetically active radiation (PAR) in this part of the tunnel but differences in photosynthesis had never actually been measured. Overall these considerations gave rise to the three results chapters seen in this project: field work to explore species-specific factors in strawberry photosynthesis, transformation of strawberry with selected genes of interest and genotyping and phenotyping of those transformed lines.

6.2 – Summary of Work

6.2.1 – Using polytunnel row position to explore photosynthetic bottlenecks in strawberry

Polytunnels for strawberry production were built along a north-south axis as part of the WET Centre (https://www.thewetcentre.com/), a project designed to study how water efficiency could be improved in soft fruit production. As part of this, fruit yield was recorded in a row by row basis and the unusual pattern described above was found and attributed to increased PAR. This presented an ideal opportunity to not only investigate this hypothesis but to examine what factors most strongly affect photosynthesis in strawberry, since understanding physiological bottlenecks may inform genetic improvement strategies.

As anticipated, capacity for carbon assimilation of Row 4 plants was heightened by up to 20 % compared to other rows in the mornings of the early season at saturating light and matched assimilation of Row 6 plants in the afternoons. This is understandable, since by the afternoon more PAR is available for Row 6 due to the position of the sun. By the mid-season however this effect was lost. This was possibly due to increased leaf age reducing photosynthetic efficiency of the leaves; chlorophyll fluorescence imaging has previously revealed strawberry leaves greater than seven weeks old have significantly dampened (~32 %) net photosynthetic rate compared to 4 – 6 week old leaves (Keutgen *et al.*, 1997). Therefore Row 4 plants were unable to fully exploit photosynthesis as they had in the early season with younger leaves. This effect may also have been caused by increased canopy size leading to greater shading within the row (Li and Yang, 2015), negating any positive effects on photosynthesis of increased PAR. Central rows received significantly greater PAR than external rows throughout the growing season. This may have contributed to greater synthesis of biomass, aiding in more rapid leaf emergence in the colder spring weather, in turn enabling greater total photosynthesis to be performed and greater exploitation of the higher PAR

observed; this has previously been posited as an improvement to canopy architecture development that could be used to increase carbon assimilation (Murchie *et al.*, 2009).

Intrinsic water use efficiency (*WUEi*) was also examined to better understand strawberry stomatal dynamics in the field. This gave a surprising insight. While Row 4 strawberries were capable of performing greater photosynthesis at saturating, there was no significant associated water cost. This suggests that photosynthesis was performing more efficiently as a result of photochemistry and not from the opening of stomata to allow more carbon into the mesophyll of the leaf. It is therefore apparent that Row 4 photosynthesis was limited by stomatal aperture and that conservation of water within strawberry is a strong driving factor affecting how much photosynthesis is performed. This association between increased PAR. decreased *gs* and regulation of photosynthetic rate has previously been described (Choi, 2021; Kanno *et al.*, 2022) and this work provides an explanation for that relationship. Manipulation of stomatal dynamics in strawberry therefore represents an excellent target for improving strawberry photosynthesis and yield. Despite this stomatal limitation, Row 4 plants still outperformed other rows in terms of photosynthetic efficiency. This demonstrated that strawberry photosynthesis could still be improved and that this photosynthesis wasn't entirely sink-limited. This agrees with research exploring the effect of runner and leaf removal on strawberry yield which found that removal of runners (i.e. sinks) increased fruit yield while removal of leaves (i.e. sources) decreased fruit yield (Sønsteby *et al.*, 2021).

The measurements carried out to record carbon assimilation and *WUEi* were performed using a single ADC Infrared Gas Analyser (IRGA) over a period of several days and each light response curve took ~50 minutes to record. To minimise day-to-day differences in weather and differences between different times of day, measurements were alternated between rows in a randomised-block design. This experiment would be improved however if multiple machines could have been used. While this would have introduced error from differences in individual

IRGAs' measurements of gas exchange, it would have minimised greater error arising from time of day. With the same number of measurements taken over a smaller time scale, differences between, for example, early morning and late morning measurements could have been examined and outliers more easily removed from the dataset. The use of more machines could also have increased the number of repeats, further improving validity. Unfortunately, this work was limited by availability of a single IRGA. This also limited the work to only one polytunnel. Measuring this effect across multiple polytunnels would have given more technical repeats to further improve the robustness of these findings.

Row 4 plants were found to produce the greatest mass of strawberry fruits and this increase was derived from an increase in fruit number. Increased photosynthesis has previously been shown to accelerate developmental rate (Simkin *et al.*, 2017b) so Row 4 strawberries producing greater numbers of floral initiations, and therefore more fruits, is a likely outcome. A less predictable outcome however was internal edges of western rows yielding greater numbers of fruits. This may be explained by a warmer microclimate from heat radiation of adjacent rows, since in high light (such as that experienced by polytunnel grown strawberries during summer) strawberry photosynthesis is limited by temperature (Rivero *et al.*, 2021).

Improving availability of PAR across a polytunnel, removing limitations imposed by stomatal aperture and increasing efficiency of photochemistry appear to be three strong approaches that could be used to increase strawberry photosynthesis and fruit yield. Applying this in the field could involve growing strawberries on slopes to reduce row shading and increase PAR for every row (as seen in commercial grape production) to maximise quality and yield (Mania and Guidoni, 2018)) or the provision of supplemental LED lighting to stimulate photosynthesis (Yang *et al.*, 2022). Importantly, it must be considered how these changes would be applied to polytunnel grown strawberry versus glasshouse grown strawberry. In this experiment, strawberries were grown in a typical commercial field environment, exposed to the

elements and “pestered” by environmental fluctuations and pests and diseases. However, an increasing amount of strawberry production is occurring inside in “pampered” conditions, protected from sudden changes in environmental conditions and more defended from pests. A particularly relevant example to this thesis is that crops grown under stable light indoors are protected from sunflecks unlike crops grown in the field, requiring less energy to be diverted towards photoprotection to prevent the much more energy costly process of photoinhibition (Murchie and Niyogi, 2011). This is also relevant to consider given the rise of growing strawberries in vertical farms under LED lights, which could lead to a 30 x increase in yield per capita without using up arable land (Despommier, 2013). It is clear that different approaches may be required for field-grown versus glasshouse grown crops. It is a well-documented phenomenon that strategies to increase photosynthetic efficiency are often effective in glasshouse conditions but do not translate to yield increases in the field (Sinclair *et al.*, 2019) and when yield increases are seen they are often inconsistent between trials (likely due to a large effect of weather) (López-Calcagno *et al.*, 2020). Despite this, where increases have been seen in the field, they have been related to improvement of mechanisms that limit damage to the chloroplast and photosynthetic apparatus, namely photorespiration (Lopez-Calcagno *et al.*, 2018) and photoprotection (Ahmad *et al.*, 2020). It is therefore apparent that approaches to improve photosynthesis in field grown crops need to focus on strategies to prevent energy-costly damage experienced a side effect of photosynthesis, rather than direct improvement of photosynthetic efficiency (as can effect large yield changes in controlled conditions).

For genetically improved strawberries, since current regulations ban growth of genetically modified (GM) crops for human consumption, the law would need to be altered or rescinded before these could be grown in a commercial setting in the United Kingdom (Environmental Protection Act 1990, 1990). Global opinion on GM foods remains divided, with the European Union (EU) particularly against their use. In fact, the EU’s new “Farm-To-Fork” strategy as part of the European Green Deal would instead seek to increase organic farming

practices typically associated with reduced productivity rather than embrace GM crops as a tool to increase land use efficiency and combat climate change (Kovak *et al.*, 2022). Attitudes towards GM foods are more positive in the United States of America (USA), but the proportion of consumers that feel GM foods are safe (37 %) still remains a minority of the population (Sendhil *et al.*, 2021). Despite this, GM foods are legal and recently a transgenic tomato fruit accumulating high concentrations of health promoting anthocyanins was approved for commercial exploitation (APHIS, 2022), suggesting that the GM food market will continue to grow in the USA.

6.2.2 – Transformation of cultivated strawberry

The work carried out in Chapter 3 demonstrated that increased availability of PAR led to an increase in photosynthetic efficiency and fruit yield, demonstrating that strawberry photosynthesis is not yet fully optimised. Therefore, an effort was made to improve strawberry photosynthetic efficiency. Two genes were selected for study: Calvin-Benson-Bassham Cycle (CBB Cycle) enzyme sedoheptulose-1,7-bisphosphatase (SBPase), for its extensive research history in photosynthetic improvement (Lefebvre *et al.*, 2005; Driever *et al.*, 2017; Simkin *et al.*, 2015), and starch synthesis adenosine diphosphate glucose pyrophosphorylase (AGPase), for its predicted limitation on photosynthesis (Zhu *et al.*, 2007) and to reduce any potential effects of sink-limited growth.

Plasmids containing these genes of interest were either donated by the University of Essex or generated via Golden Gate Assembly. Plasmids were originally to be constructed via the pENTR/TOPO-D Cloning Kit (Invitrogen) but attempts were unsuccessful. The donated and

constructed plasmids contained hygromycin or glufosinate resistance genes for selection of transformed callus tissue.

Transformation of strawberry ultimately proved highly complex. The cultivar of choice, Calypso, proved to have poor tolerance for hygromycin. The concentration of hygromycin used in this thesis, 25 mg L^{-1}, was not outside the normal level used in transformation of plant material, or indeed in transformation of strawberry, and was designed to prevent the formation of genetic chimeras. One study used up to 50 mg L^{-1} hygromycin and were still successful (Mathews, Wagoner, Kellogg, *et al.*, 1995), suggesting Calypso has a poor tolerance to hygromycin compared to the cultivars tested (Tristar and Totem). Unfortunately for this work, the selected concentration significantly retarded growth and development of the transformed tissue. Furthermore, experiments using glufosinate as a selectable marker killed all tissue at concentrations as low as 3 mg L^{-1}, indicating that cultivated strawberry has an extraordinarily low tolerance for this compound.

If this work could be repeated, kanamycin (Schaart *et al.*, 2004b) would be chosen as the selectable marker for transformation of strawberry and projects building on these findings (as part of the Collaborative Training Partnership for Fruit Crop Research and South Coast Biology DTP) are already employing this method. Despite these setbacks, transformed plants were still successfully generated but only for the plasmid containing coding sequences for both SBPase and AGPase and for plants containing kanamycin as the selectable marker for different projects.

As part of efforts to increase the speed and efficiency of this protocol, the AGL1 and EHA105 strains of *Agrobacterium tumefaciens* were tested to investigate if bacterial strain affected transformation efficiency. Despite being closely related genetically (De Saeger *et al.*, 2021), AGL1 was significantly better at inducing callus in the presence of a selectable marker than EHA105. This is an understudied area and is, to the author's knowledge, the first instance

of bacterial strains being compared to understand changes in the efficiency of the transformation protocol in strawberry.

The final aspect as this work was the analysis of transgenic strawberry plants to confirm successful transformation. This required a little groundwork. Due to the high concentration of phenolic compounds found in strawberry leaves (Giampieri *et al.*, 2014), RNA extraction can be compromised. To ensure RNA extraction of transgenic plants would go smoothly an RNA kit was tested in advance. The NucleoSpin RNA Plant and Fungi Kit (Macherey-Nagel) was identified as a phenol-free method for extracting large quantities of high quality RNA from strawberry leaf. Further to this, it was identified that four week old leaves produced better quality RNA than eight week old leaves. Since experiments were carried out on four week old daughter plants, this result was ideal.

To identify successful transformation and expression of transgenes, RT-qPCR was carried out on transgenic lines and compared to a wild type (WT) control. In most instances, a 1 – 2 fold increase in expression was found for *Arabidopsis* SBPase and AGPase relative to the average expression of three selected housekeeping genes. There were two instances where this what not the case. Firstly, the WT showed no expression of the *Arabidopsis* RNA, indicating that the primers only captured expression of the transgenes and not of any homologous sequences in the strawberry genome. Secondly, line 45 showed no expression of SBPase. This suggests that, despite being transformed with a plasmid containing both AGPase and SBPase coding sequences, only AGPase was successfully expressed. This could be caused by transgene silencing, failure of the gene to fully insert or a position effect of the inserted transgene (Halpin, 2005). This turned out to be a stroke of good fortune, as this line could be taken forward as overexpressing AGPase only. This enabled comparison of WT, AGPase overexpressing (A) and SBPase/AGPase co-overexpressing (SA) lines to better unpick the individual contributions overexpressing AGPase and SBPase make to the photosynthetic phenotype of transformants.

To improve this work, Western blotting should be carried out with SBPase and AGPase specific antibodies. This would provide concrete evidence that overexpression of these RNAs is in fact leading to increased quantities of protein, thereby explaining any phenotypic differences from the WT control. Unfortunately at the time this was to be carried out, all plants being used for analysis were damaged by a suspected infection and any results obtained would have been unreliable. This also reduced capacity for certain phenotyping experiments which is discussed below.

6.3.3 – Studying genetically enhanced photosynthesis in strawberry

Four-week old daughter plants were severed from their mother plants and taken to the University of Essex for chlorophyll fluorescence analysis. There, it was observed that the SA line had an increased photosynthetic efficiency of photosystem II (PSII) relative to both the WT and A lines. This implicated SBPase as a rate-limiting enzyme in strawberry photosynthesis and suggested that AGPase did not limit photosynthetic rate in strawberry. However, the greater photochemical quenching (qP) seen in the A and SA lines relative to the WT control demonstrated that line A maintained the same number of open reaction centres as line SA, despite SA performing photosynthesis at a greater rate. This was caused by an increase in non-photochemical quenching (NPQ) diverting energy away from the electron transport chain. It is worth noting here that the WT control was closing more reaction centres but not outperforming the SA or A lines in operating efficiency of PSII. This may suggest that AGPase is in fact rate-limiting on strawberry photosynthesis, but other limiting factors prevent any increase in photosynthetic efficiency from being realised. Increased qP and NPQ, as seen in the A and SA lines, are correlated with increased fruit yield in strawberry (Choi, 2021).

To better investigate if the A and SA lines have an enhanced photosynthesis phenotype, gas exchange measurements should be carried out. Since SBPase is a CBB Cycle enzyme and AGPase uses products of the CBB Cycle, understanding how overexpression of these enzymes affects the uptake of carbon is necessary for determining if photosynthesis has truly been improved as implicated from the chlorophyll fluorescence imaging. This plan couldn't be carried out in time for this project's completion due to plant health as noted above. Further to this, collection of vegetative or fruit yield data would have further contributed valuable data to this project, since this is the end goal of enhanced photosynthesis.

It would also be pertinent to collect fruit flavour and quality data. One of the main reasons to enhance strawberry photosynthesis is to increase the nutritional and flavour quality of fruits in a similar manner to that seen under *e*[CO_2] (Wang *et al.*, 2003; Wang and Bunce, 2004); as such, this should be studied alongside fruit yield. This is particularly interesting in the context of AGPase. AGPase is essential for the synthesis of starch (Fünfgeld *et al.*, 2022) and sugar accumulation in strawberry fruits is driven by a breakdown of accumulated starch (with an estimated 3 % of this derived from transitory starch) (Souleyre *et al.*, 2004). The high sugar content of strawberry fruits is essential to their pleasant flavour and represents up to 90 % of the soluble solid content of the fruit (Perkins-Veazie, 2010). Manipulation of starch metabolism therefore represents a target in strawberry for both improved photosynthesis (and by extension, improved fruit yield) and improved flavour. Traditional breeding has previously struggled to simultaneously amplify both of these traits and has focussed more on increasing yield (Cockerton *et al.*, 2021) so identifying genetic targets that could increase both fruit yield and quality is an exciting prospect.

These genetic targets could be applied to either genetic manipulation or to traditional breeding. It is well understood that different cultivars display different photosynthetic rates, but the effect of these phenotypes on yield is complicated by how responsive these varieties are to

fluctuations in environment; this may cause energetic inefficiencies associated with inappropriate stomatal aperture, lack of photoprotection and ability to maximise photosynthesis when it is most needed for growth and development (i.e. during fruiting) (Hancock *et al.*, 1989). Further to this, genetic variation in strawberry photosynthesis between these cultivars has not been explored. It is likely that, from a genetic perspective, how strawberry photosynthesis translates into yield is controlled by a large number of alleles (since environmental response has a large impact on net energy gain), making the improvement of strawberry photosynthesis through breeding a tricky task. One possible area that could be tackled is increasing the concentration of strawberry carotenoids. Carotenoids confer photoprotective properties to green tissue and provide essential nutrients when consumed (e.g. pro-vitamin A) (Barbey *et al.*, 2020). Increasing their concentration throughout strawberry leaf and fruit tissues could therefore improve photosynthesis and yield through improved photoprotection while simultaneously enabling the production of strawberry fruits with greater health benefits for consumers. Quantitative trait loci (QTLs) for PHYTOENE SYNTHEASE and Z-CAROTENE DESATURASE have been identified that explain 50 % – 62 % of carotenoid biosynthesis flux in mature fruits (Fraser *et al.*, 2007). Exploiting such QTLs in marker assisted breeding programmes could lead to improvement of strawberry photosynthesis and yield without requiring genetic modification and its associated legal issues.

6.3 – Challenges and Obstacles

Numerous challenges have been presented over the course of this project, most notably the COVID-19 pandemic. The abrupt closure of work and loss of facilities significantly delayed the transformation workpackage of this project, an already challenging step due to issues with retarded regeneration rates and the revealed toxicity of the selectable markers. These delays also presented problems during phenotyping, since they delayed work to a point where, when extensive plant health issues arose, no further phenotyping could be carried out in the remaining time. A significant number of transformed plants remain in tissue culture, so the resources generated in this project have not been lost, but there was insufficient time to wean these onto soil for analysis. Despite this, the project has still yielded interesting, good quality and informative results that can be taken forward for use in other work.

6.4 – Future Directions

This project has opened many new avenues of scientific exploration. From the field work, repetition in a second season is necessary to measure the significance of variation in weather between years. This would ensure that the measured results were not a unique product of the summer weather in 2021. Additionally, it would be interesting to test more patterns across the tunnel. The increase in yield seen in Row 4 was only about 3 % more compared to Row 3, whereas historical data shows Row 4 consistently yielding 10 – 15 % more fruit than all other rows. Yield and photosynthesis measurements were carried out in the centre of the polytunnel to avoid edge effects; could this increase in Row 4 yield be more pronounced at the polytunnel ends? Measuring how fruit yield and photosynthesis vary on a north-south axis as well as an east-west axis may provide insight into this question. A further question asked about fruit yield involves microclimates between rows. If there is a slight temperature difference in the air between rows, could this create a more favourable growth condition and effect greater fruit production? This would be the first step in exploring the hypothesis that yield within a row is greater on the less exposed edge.

For the transformation work, different cultivars should be tested. Calypso is an outdated cultivar no longer exploited by industry and while it is useful for transformation work as a proof of concept, it is important that any obtained results can be replicated in elite breeding germplasm. Future work should test the regeneration capacity of elite varieties of strawberry to find a commercially relevant variety that can be used in transformation experiments or included within breeding populations for the development of new varieties for the future. This testing of elite varieties was last carried out almost 20 years ago (Passey *et al.*, 2003) and while this research still provides relevant insight into how different strawberry cultivars respond to different regeneration media, a modern update would be of great use to the strawberry research community. Elite lines should also be tested for the relationship between their

photosynthesis and yield characteristics via high throughput phenotyping and genotyping to identify QTLs associated with increased photosynthetic efficiency. These lines should then be prioritised in breeding programmes and future genetic manipulation work in strawberry to see if genetic enhancement of photosynthesis translates into elite strawberry cultivars. Transformation should also be tested with different *Agrobacterium* strains with more genetic diversity. The work carried out here demonstrates even closely related strains have significantly different efficiencies for successfully transforming strawberry, representing an as yet untapped well of possibility for increasing the efficiency of the strawberry transformation process. Finally, different genes of interest should be tested. There are so many approaches that can be used to increase photosynthesis (as reviewed in Chapter 1) and while it appears overexpression of SBPase and AGPase is a successful approach in strawberry, other methods may be more successful at increasing photosynthesis. For example, increasing the number of stomata by overexpression of the STOMAGEN gene may overcome stomatal limitations described in Chapter 3 and has been shown to elevate photosynthesis in *Arabidopsis* (Tanaka *et al.*, 2013). It is possible that this will be particularly impactful on strawberry photosynthetic rate due to its limitations from stomatal aperture. Future work should focus on understanding which genetic manipulations are best suited for application in strawberry and continue expanding the field to other high-value fruiting crops such as tomato. With this approach, genetically enhanced photosynthesis could be used to increase production across all agricultural sectors and work towards improved global food security.

Chapter 7 – References

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Chapter 8 – Appendixes

Displayed here are additional data, figures and information pertinent to the text.

Crop	CO_2 Treatment	Additional Treatment(s)	Fruit Yield	Fruit Quality	Reference
Tomato	900 ppm	100 µmol s^{-1} m^{-2} supplementary lighting	12 % – 15 % increase in marketable fruit yield under supplementary lighting, 7 % increase in marketable fruit yield in absence of additional treatment.	N/A	(Fierro et al., 1994)
	700 ppm – 1000 ppm	N/A	32 % increase in marketable fruit yield.	N/A	(Hartz et al., 2019)
	700 ppm	Doubled N fertilisation	N/A	13 % – 25 % decrease in fruit lycopene content across harvests with elevated [CO_2]. 9 % increase in fruit lycopene content with increased N fertilisation.	(Helyes et al., 2011)
	900 ppm	N/A	30 % increase in marketable fruit yield.	N/A	(Hinckleton and Joliffe, 1978)
	700 ppm – 900 ppm	N/A	~30 % increase in individual fruit weight.	~18 % increase in vitamin C. ~Up to 20 % reduction in major acids (citric, malic, oxalic). ~45 % increase in monomeric sugars (glucose, fructose).	(Islam et al., 1996)
	1000 ppm	N/A	74.27 % – 83.61 % increase in tomato fresh weight per plant.	16.12 % – 20.85 % increase in total sugars. 20.02 % – 24.72 % decrease in vitamin C. 4.79 % – 6.76 % decrease in total acids.	(Khan et al., 2013)
	650 ppm and 1000 ppm	N/A	17 % increase in fruit yield at 650 ppm and 48 % increase in fruit yield at 1000 ppm.	N/A	(Kimball and Mitchell, 1978)
	1200 ppm	Salinity up to 4.58 x control = 1.53 dS m^{-1}	> 40 % loss in dry fruit yield at highest salinity treatment completely offset by increased [CO_2].	Increased salinity and [CO_2] combined increases total sugar and acid content of fruits by up to ~30 %.	(Li et al., 1999)
	700 ppm	UV-B exposure up to 1.744 kJ m^{-2}	38 % increase in fruit yield in absence of additional UV-B treatment, up to 46 % increase in fruit yield with UV-B treatment.	Up to ~22 % increase in soluble sugars, ~24 % increase in organic acids, ~40 increase in vitamin C and ~47 % increase in lycopene content of tomato fruits grown under increased [CO_2] and UV-B treatment.	(Li et al., 2007)
	800 ppm	0 – 0.5 g N kg^{-1} soil and soil water content 25 % – 35 % v/v	Across all treatments, -3.3 % – 28 % increase in total fruit yield.	-17.9 % – 11.9 % increase in total fruit sugars and -18.9 % – 12.7 % increase in total fruit acids across all treatments.	(Liu et al., 2020)

	550 ppm and 700 ppm	N/A	54 % increase in fruit yield at [CO_2] = 550 ppm and 125 % increase in fruit yield at [CO_2] = 700 ppm.	1.4 % – 11.4 % decrease in total soluble solids, 27.3 % – 31.8 % decrease in total acids and 16.1 % – 29.0 % increase in vitamin C.	(Mamantha et al., 2014)
	510 ppm	N/A	9.9 % increase in fruit yield.	N/A	(Nederhoff, 1994)
	1000 ppm	N/A	30 % increase in total fresh fruit yield per plant.	N/A	(Nilsen et al., 1983)
	1000 ppm	N/A	43 % increase in total fruit yield.	No significant effect on fruit quality parameters.	(Özçelik and Akilli, 1999)
	590 ppm	70 % irrigation of control and programmed root drying	Fruit dry weight not significantly affected by [CO_2] across all irrigation treatments.	N/A	(Pazzagli et al., 2016)
	Exact increase not given	N/A	~23 % – 46 % increase in yield across 4 different cultivars.	N/A	(Peet and Willits, 1984)
	550 ppm and 700 ppm	+2 °C increase in temperature	18.37 % – 21.41 % increase in fruit yield due to increased [CO_2].	Up to 10 % increase in total sugars, 44 % increase in vitamin C and 32 % increase in lycopene content at elevated [CO_2] in absence of other treatments. Increased [CO_2] rescues reduction in quality derived from increased temperature.	(Rangaswamy et al., 2021)
	375 ppm – 675 ppm	Ozone treatment 80 nmol mol^{-1}	24 % increase in mature fruit yield across all [CO_2] treatments. 31 % decrease in fruit yield when exposed to ozone. Ozone and CO_2 treated fruit yields were not significantly different to plants grown in ambient conditions.	N/A	(Reinert et al., 1997)
	800 ppm	Salinity treatments at 5 – 7 dS m^{-1}	13 % increase in marketable fruit yield in carbon-enriched atmosphere and 31 % reduction in marketable fruit yield in increased salinity.	7 % increase in total soluble solids and no significant change in citric acid content of mature fruits. Organoleptic qualities of tomatoes grown under increased salinity and CO_2 found more preferable in sensory trials.	(Sánchez-González et al., 2016)

	1000 ppm	N/A	15.6 % increase in mean total fruit yield across 8 different cultivars.	N/A	(Tripp et al., 1999)
	800 ppm	100 or 200 mg N kg^{-1} soil, 70 % irrigation of control and programmed root drying	8 % increase in fresh fruit yield with increased [CO_2].	No significant difference in total sugars, organic acid or fruit firmness for fruits grown in elevated [CO_2].	(Wei et al., 2018)
	400 ppm – 10,000 ppm	N/A	N/A	Cultivar dependent variation in proximate composition of tomatoes and inorganic nutrient content of fruits. Increasing CO_2 correlated strongly with increased fruit calcium concentration.	(Wheeler et al., 1997)
	900 ppm	N/A	22 % increase in total fruit yield for plants grown in elevated atmospheric [CO_2].	N/A	(Yelle et al., 2019)
	800 ppm – 900 ppm	N/A	N/A	~28 % increase in vitamin C at ripe stage, ~8 % increase in total soluble solids and no significant difference in total acids. Marked preference in sensory trials for fruits grown under enriched [CO_2].	(Zhang et al., 2014)

Supplemental Table 1: Effects of elevated [CO_2] on tomato fruit yield and quality.

Crop	CO_2 Treatment	Additional Treatment(s)	Fruit Yield	Fruit Quality	Reference
Sweet Pepper	700 ppm	N/A	18 % – 22 % increase in total fruit yield.	N/A	(Aloni and Karni, 2002)
	750 ppm	N/A	19 % increase in fruit yield.	N/A	(Alonso et al., 2012)
	10,000 ppm	N/A	20 % increase in fruit yield.	N/A	(Enoch et al., 1970)
	900 ppm	N/A	7 % increase in early yielding fruits, no significant change in total fruit yield.	N/A	(Fierro et al., 1994)
	1000 ppm	N/A	51 % – 370 % increase in fruit weight per plant.	N/A	(Milhet and Costes, 1975)
	350 ppm and 450 ppm	N/A	12.9 % increase in fruit yield at $[CO_2]$ = 350 ppm and 47.4 % increase in fruit yield at $[CO_2]$ = 450 ppm.	N/A	(Nederhoff, 1994)
	700 ppm	High/low irrigation and nitrogen treatments	Fruit yield for elevated $[CO_2]$ increased with irrigation with no significant difference in fruit yield at lowest irrigation.	N/A	(Peñuelas et al., 1995)
	400 ppm and 800 ppm	Saline treatment 20 mmol L^{-1} NaCl, foliar calcium treatment 4.3 mg L^{-1}	18.9 % increase and 26.6 % increase in marketable fruit yield at 400 ppm and 800 ppm respectively relative to yield at ambient $[CO_2]$ in absence of other treatments. Foliar calcium application had little significant impact on yield. Elevated $[CO_2]$ rescued total yield loss from increased salinity.	Little significant effect of increased $[CO_2]$ on fruit inorganic nutrients or fruit colour.	(Piñero et al., 2018)
	800 ppm	Nitrogen source and saline treatment (8 and 25 mM NaCl)	8 % and 22 % increase in marketable fruit yield under salinity stress and unstressed respectively.	N/A	(Porras et al., 2017a)

	800 ppm	Nitrogen source and saline treatment (8 and 25 mM NaCl)	23 % and 29 % maximum increase in daily fruit harvest yield for 2 different nitrogen sources at low salinity.	N/A	(Porras et al., 2017b)
	367 ppm – 1000 ppm	Range of irrigation regimens	Yield increased with irrigation and carbon dioxide with a maximum yield increase with both treatments of 264 %.	N/A	(Rezende et al., 2003)
Chili pepper	1000 ppm	N/A	43.80 % – 59.55 % increase in fruit fresh weight per plant across 5 cultivars.	Up to ~15 % increase in total fruit sugars. 11.84 % – 15.84 % decrease in fruit vitamin C, non-significant decrease in fruit titratable acids. Variable effects on inorganic nutrient concentrations. Fruit amino acids and fatty acids mostly reduced.	(Azam et al., 2017)
	380 ppm – 750 ppm	+2 °C and +4 °C temperature elevation	Up to 41.9 % increase in fruit diameter under both increased carbon dioxide and increased temperature.	27 % – 44 % increase in capsaicin across all treatments for 2 cultivars across 2 growth years.	(Das et al., 2016)
	380 ppm – 750 ppm	+2 °C and +4 °C temperature elevation	53.8 % increase in fruit number per plant for $[CO_2]$ = 550 ppm and ambient + 2°C temperature, 12.3 % decrease in fruit number per plant for $[CO_2]$ = 750 ppm and ambient + 4°C temperature.	N/A	(Das et al., 2020a)
	380 ppm – 750 ppm	+2 °C and +4 °C temperature elevation	Up to ~140 % increase in fruit yield per plant for $[CO_2]$ = 550 ppm and ambient + 2°C temperature, up to ~36 % reduction in fruit yield per plant for $[CO_2]$ = 750 ppm and ambient + 4°C temperature.	N/A	(Das et al., 2020b)
	380 ppm – 1140 ppm	N/A	Up to 88.5 % increase in number of fruits per plant, up to 13.0 % increase in fruit length, up to 15.0 % increase in fruit width and up to 14.3 % increase in pericarp thickness.	N/A	(Garruña-Hernández et al., 2012)
	380 ppm – 1140 ppm	N/A	Up to 142 % increase in fruit yield (g $plant^{-1}$). Fruit size and number same as reported in Garruña-Hernández et al., 2012.	No change in colour of ripe fruits. Up to 28.6 % increase in capsaicinoids in ripe fruit.	(Garruña-Hernández et al., 2013)

	400 ppm – 900 ppm	Natural light (233 µmol m^{-2} s^{-1}) and supplementary light (463 µmol m^{-2} s^{-1})	92 % – 113 % increase in total fruit yield per plant across all CO_2 treatments with supplementary lighting relative to ambient control at 400 ppm. 47 % – 113 % increase in total fruit yield per plant across all CO_2 treatments with natural lighting relative to ambient control at 400 ppm.	2 % – 10 % decrease in soluble sugars and 13 % – 34 % decrease in vitamin C in elevated [CO_2]. 61 % increase in capsaicin at [CO_2] = 550 ppm, however 49 % – 61 % decrease in capsaicin for [CO_2] > 550 ppm.	(Li et al., 2017)
Eggplant	200 ppm – 3000 ppm	N/A	209 % increase in fruit fresh weight and 134 % increase in fruit dry weight.	N/A	(Imazu et al., 1967)
	1000 ppm	N/A	31 % increase in fruit yield across a full year of harvests.	N/A	(Milhet and Costes, 1975)
	663 ppm	N/A	23.6 % increase in fruit yield.	N/A	(Nederhoff, 1994)

Supplemental Table 2: Effects of elevated [CO_2] on fruit yield and quality of solanaceous fruiting crops.

Crop	CO_2 Treatment	Additional Treatment(s)	Fruit Yield	Fruit Quality	Reference
Strawberry	400 ppm, 650 ppm and 900 ppm	Ambient temperature (25 °C) and elevated (30 °C)	9.9 % – 33.4 % increase in total fruit yield at ambient temperature for cultivar "Albion", 0.9 % – 31.2 % decrease in total fruit yield at ambient temperature for cultivar "San Andreas". Elevated [CO_2] rescues yield loss from elevated temperature.	N/A	(Balasooriya et al., 2018)
	400 ppm, 650 ppm and 900 ppm	Ambient temperature (25 °C) and elevated (30 °C)	N/A	Total fruit polyphenolic content, flavonoid content, monomeric anthocyanin content and antioxidant content increased in correlation with elevated [CO_2] at both temperatures for multiple cultivars (~9 % – ~325 % increase overall increase at [CO_2] = 900 ppm).	(Balasooriya et al., 2019)
	600 ppm – 1000 ppm	N/A	62 % increase in total fruit yield in elevated [CO_2].	N/A	(Bushway and Pritts, 2002)
	553 ppm	Nitrate treatment (4 x 10^{-2-0} mM)	42 % increase in fresh fruit yield in elevated [CO_2] at high N, 17 % increase in fresh fruit yield in elevated [CO_2] at low N.	N/A	(Deng and Woodward, 1998)
	900 ppm, 1500 ppm, 3000 ppm	N/A	31 %, 43 % and 51 % increase in fruit yield at 900 ppm, 1500 ppm and 3000 ppm respectively.	N/A	(Enoch et al., 1975)
	700 ppm – 1000 ppm	N/A	5.4 % and 12.7 % increase in marketable fruit yield for cultivars "Irvine" and "Chandler" respectively.	N/A	(Hartz et al., 2019)
	720 ppm	5 °C increase in temperature, nitrate treatment (50 mL 0.1 % NH_4NO_3 twice per week)	~120 % increase in total fruit dry weight in elevated [CO_2], ~73 % increase in total fruit dry weight in elevated [CO_2] with nitrogen treatment. No significant change in fruit yield for all other treatments individually and in combination.	48 %, 21 %, 36 % and 18 % decrease in fruit anthocyanin content, total phenolic content, total flavonoid content and total antioxidant content respectively at elevated [CO_2]. 29 % and 35 % increase in fruit fructose and glucose respectively. 43 % increase in total sugars.	(Sun et al., 2012)

	1000 ppm	N/A	47 % increase in fruit number per plant, no significant change in individual fruit weight.	N/A	(Sung and Chen, 1991)
	Ambient + 300 ppm, Ambient + 600 ppm	N/A	17.6 % and 38.5 % increase in individual fruit weight at [CO_2] = ambient + 300 ppm and [CO_2] = ambient + 600 ppm respectively.	7.0 % – 25.9 % increase in glucose, fructose and sucrose. 5.2 % – 47.4 % decrease in citric, malic and quinic acids. Stepwise increase in concentration of most key volatile esters and up to 115.0 % and 149.6 % increase in fruit furaneol and linalool content.	(Wang and Bunce, 2004)
	Ambient + 300 ppm, Ambient + 600 ppm	N/A	N/A	13.3 % increase in fruit ascorbic acid. Stepwise increase in antioxidant and flavonoid compounds with increasing carbon dioxide.	(Wang et al., 2003)
Raspberry	436 ppm	N/A	12 % increase in total berry yield and 5 % increase in average individual berry weight.	N/A	(Mochizuki et al., 2010)
Nashi Pear	700 ppm	Ambient + 4 °C temperature	16.6 % increase in fruit weight with elevated [CO_2]. Elevated [CO_2] rescues yield loss from increased temperature.	Up to 15.9 % reduction in fruit firmness with elevated [CO_2]. Up to 22.5 % increase in total soluble solids with no significant change in acidity with elevated [CO_2].	(Han et al., 2012)

Supplemental Table 3: Effects of elevated [CO_2] on fruit yield and quality of rosaceous fruiting crops including cultivated strawberry.

Crop	CO_2 Treatment	Additional Treatment(s)	Fruit Yield	Fruit Quality	Reference
Cucumber	400 ppm, 625 ppm, 1200 ppm	2 mmol L^{-1}, 7 mmol L^{-1}, 14 mmol L^{-1} NO_3^-	Up to 73 % increase in fresh fruit yield for plants grown at highest [CO_2] versus plants grown at lowest [CO_2] at greatest N fertilisation. No significant difference in yield for lower N fertilisation.	N/A	(Dong et al., 2017)
	400 ppm, 800 ppm, 1200 ppm	0.06 g N kg^{-1} soil (low N), 0.24 g N kg^{-1} soil (high N)	31 % – 37 % increase in fresh fruit yield for [CO_2] = 800 ppm and 1200 ppm at low N. 71 % – 106 % increase in fresh fruit yield for [CO_2] = 800 ppm and 1200 ppm at high N.	Across both nitrogen treatments at [CO_2] = 1200 ppm, fruit fructose was increased by 5 % – 6 %, fruit glucose was increased by 10 % – 12 % and fruit starch was increased by 29 % – 40 %.	(Dong et al., 2018a)
	400 ppm, 625 ppm, 1200 ppm	2 mmol L^{-1}, 7 mmol L^{-1}, 14 mmol L^{-1} NO_3^-	N/A	75 % increase in fruit fructose and 73 % increase in fruit glucose at 7 mmol L^{-1} at highest [CO_2]. No significant change in fruit titratable acidity. Elevated [CO_2] reduced dietary fibre by 13 % – 18 % across all fertilisation treatments. Up to 84 % reduction in fruit nitrogenous compounds in elevated [CO_2] across all nitrogen treatments.	(Dong et al., 2018b)
	900 ppm, 1500 ppm, 3000 ppm	N/A	18.4 % – 26.3 % increase in fresh fruit yield across all CO_2 elevations.	N/A	(Enoch et al., 1975)
	700 ppm – 1000 ppm	N/A	20 % – 30 % increase in marketable fruit yield across two growing seasons.	N/A	(Hartz et al., 2019)
	150 ppm, 245 ppm, 430 ppm, 790 ppm, 1500 ppm, 2870 ppm	N/A	15 % – 36 % increase in fresh fruit yield for elevated [CO_2] relative to [CO_2] = 245 ppm. 38 % decrease in fresh fruit yield at [CO_2] = 150 ppm relative to [CO_2] = 245 ppm.	N/A	(Heij and van Uffelen, 1984)

	380 ppm	N/A	35 % increase in fresh fruit yield in greenhouse supplemented with [CO_2] versus control greenhouse.	N/A	(Klaring et al., 2007)
	900 – 1000 ppm	0.6 °C – 1.8 °C cooling	35.4 % increase in dry fruit mass in cooled and elevated [CO_2] conditions.	N/A	(Luomala et al., 2008)
	364 ppm, 620 ppm	N/A	Up to 10.2 % increase in individual fruit weight for August production in elevated [CO_2].	No significant change in fruit dry matter content.	(Nederhoff, 1994)
	1000 ppm	N/A	8.9 % increase in fruit weight but no significant change in fruit number at elevated [CO_2].	N/A	(Peet et al., 1986a)
	600 – 700 ppm	N/A	20 % increase in fresh fruit yield at elevated [CO_2].	N/A	(Sanchez-Guerrero et al., 2005)
	400 – 500 ppm	N/A	19 % increase in fresh fruit yield at elevated [CO_2].	N/A	(Sanchez-Guerrero et al., 2009)
	700 ppm	N/A	14.2 % – 18.4 % increase in fresh fruit yield at elevated [CO_2] across two crop cycles.	Overall reduction in fruit inorganic nutrients (N, P, K, Ca, Mg).	(Segura et al., 2001)
Melon	400 ppm, 800 ppm, 1200 ppm	0, 25, 50 mmol NaCl	Up to 29 % increase in fruit yield across all [CO_2] elevations at no additional salinity. Elevated [CO_2] partially rescues yield loss from salinity (by up to 18 %) but is insufficient to fully mitigate yield loss.	N/A	(Mavrogianopoulos et al., 1999)
	1000 ppm	N/A	13 % increase in muskmelon fruit number and 8 % increase in muskmelon fruit weight during summer production under elevated [CO_2].	N/A	(Milhet and Costes, 1975)
Squash	700 – 1000 ppm	N/A	15.5 % – 19.7 % increase in total marketable yield across 2 growing seasons.	N/A	(Hartz et al., 2019)

Supplemental Table 4: Effects of elevated [CO_2] on fruit yield and quality of cucurbitaceous fruiting crops.

Crop	CO_2 Treatment	Additional Treatment(s)	Fruit Yield	Fruit Quality	Reference
Grape	400 ppm and 700 ppm	Temperature ambient + 4 °C, cyclic drought	Elevated [CO_2] only resulted in significant differences in bunch fresh weight for cv. Tempranillo red at full irrigation and elevated temperature (~+35 %) and for cv. Tempranillo white at cyclic drought and elevated temperature (~+39 %). Dry weight increase for elevated [CO_2] of 2.1 % – 144.1 % across both varieties and all treatments was reported except in cv. Tempranillo red at ambient temperature where a 2.8 % – 21.7 % reduction in dry weight was reported across both irrigation regimens.	Across both cultivars (red and white Tempranillo), elevated [CO_2] reduced fruit pH by up to 11 % across all treatments and rescued reduction in total soluble solids from cyclic drought. No significant changes in malic acid, tartaric acid and total polyphenol content of fruits grown at elevated [CO_2] versus ambient [CO_2] in the absence of other treatments (though a 48 % increase in anthocyanin content was reported for fruits grown in elevated [CO_2]).	(Kizildeniz et al., 2015)
	400 ppm and 700 ppm	Temperature ambient + 4 °C, cyclic drought	Elevated [CO_2] did not significantly affect number of berries per bunch of red or white Tempranillo across all growth conditions but did rescue reduced berry number from cyclic drought. Berry fresh weight was increased in both cultivars by up to 45 % across all treatments, however most increases were not significant.	N/A	(Kizildeniz et al., 2018a)
	400 ppm and 700 ppm	Temperature ambient + 4 °C, cyclic drought	8 % higher bunch fresh weight in white Tempranillo vs red Tempranillo. 14 % reduction in yield for grapes grown under cyclic drought, 14 % increase in yield for grapes grown under elevated [CO_2].	2.6 % increase in total soluble solids for plants grown under elevated [CO_2] across all treatments, 5.3 % increase for plants grown under elevated [CO_2] in drought conditions. 7 % reduction in malic acid and 20 % increase in tartaric acid was reported across all treatment conditions for grape berries grown under elevated [CO_2].	(Kizildeniz et al., 2018b)
	700 ppm	Temperature ambient + 4 °C, 40 % irrigation	No significant effect on berry weight.	10 % increase in total soluble solids of fruits grown under climate change conditions (elevated [CO_2], temperature and partial irrigation) at 14 days and 28 days. No significant change in tartaric acid was reported, however an up to 51 % reduction in malic	(Parra et al., 2010)

				acid for berries grown under elevated [CO_2] and temperature was reported (except for berries grown under partial irrigation with high total soluble solids). No significant difference in total anthocyanins, total phenolic compounds or tonality for fruits grown under climate change conditions versus control.	
Orange	400 ppm and 700 ppm	N/A	Fruit number increased by 74 ± 9 % in elevated [CO_2] and fruit weight increased by 4 ± 2 % across 8 years of sour orange fruit harvesting.	Fruit vitamin C concentration increased by 5 ± 1 % in elevated [CO_2] across 8 years of sour orange fruit collection. Vitamin C concentration increased up to 15 % in years with high fruit number production, due to smaller fruit size.	(Idso et al., 2002)
	400 ppm and 700 ppm	N/A	Total sour orange fruit fresh yield increased by 85 % in elevated [CO_2], mostly derived from an 81 % increase in fruit number with no significant difference in individual fruit mass.	N/A	(Kimball et al., 2007)

Supplemental Table 5: Effects of elevated [CO_2] on fruit yield and quality of grape and orange (not including FACE studies on grape).

Transgene Origin	Transgene Overexpressed	Plant	Biomass and Yield	Reference
Calvin-Bensom Cycle (CBC)	SBPase	Tobacco	40 % increase in shoot biomass in glasshouse conditions.	(Lefebvre et al., 2005)
			12 % increase in shoot biomass in field at ambient [CO_2] = 385 ppm, 22.7 % increase in shoot biomass in field at elevated [CO_2] = 585 ppm.	(Rosenthal et al., 2011)
		Rice	~Up to tripling of rate of biomass increase relative to WT at 45 days of growth in elevated temperature (30 °C – 45 °C).	(Feng et al., 2007b)
		Wheat	~Up to tripling of rate of biomass increase relative to WT for transgenic plants grown at 250 mM NaCl.	(Feng et al., 2007a)
		Tomato	Up to 40 % increase in harvestable yield in glasshouse conditions.	(Driever et al., 2017)
		Arabidopsis	~30 % increase in total vegetative biomass for line with greatest biomass gains.	(Ding et al., 2016)
			42 % increase in plant biomass for transgenic plants compared to WT at growth stage when inflorescence is first visible. 14 % – 23 % increase in biomass dependant on growth conditions at seed harvest. 39 % – 53 % increase in seed yield dependant on growth conditions.	(Simkin et al., 2017)
	FBPA	Tobacco	Up to 30 % increase in biomass at ambient [CO_2] = 370 ppm, up to 120 % increase in biomass at elevated [CO_2] = 700 ppm. Grown in glasshouse conditions.	(Uematsu et al., 2012)
		Arabidopsis	42 % increase in plant biomass for transgenic plants compared to WT at growth stage when inflorescence is first visible. 25 % – 29 % increase in biomass dependant on growth conditions	(Simkin et al., 2017)

			at seed harvest. 35 % – 36 % increase in increases in seed yield dependant on growth conditions.	
	cySBPase	Tobacco	20 % increase in the rate of photosynthetic CO2 fixation and a 50 % increase in final biomass.	(Tamoi et al., 2006)
	cyFBPase	Tobacco	15 % increase in CO2 fixation rates and a 30 % increase in biomass.	(Tamoi et al., 2006)
	FS Bif	Tobacco Lettuce Soybean	50 % increase in biomass for 18-week old plants grown in hydroponic growth chambers. 60 % increase in fresh weight of 6-week old plants grown in hydroponic growth chambers. Up to 31 % reduction in seed yield for WT plants grown in elevated [CO_2] = 600 ppm and elevated temperature = ambient + 3.5 °C with no reduction for transgenic plants. Up to 9 % lower harvest index in WT plants compared to transgenic lines grown at ambient conditions. Plants grown in field.	(Miyagawa et al., 2001) (Ichikawa et al., 2010) (Köhler et al., 2017)
	TK	Tobacco Rice Cucumber	Over 50 % reduction in total plant biomass in transgenic lines. Grown in a controlled environment growth chamber. No difference between transgenic lines and WT. Grown in glasshouse conditions. Estimated total yield increase of up to 10.8 % in transgenic lines over WT. Grown in glasshouse conditions.	(Khozaei et al., 2015) (Suzuki et al., 2017) (Bi et al., 2013)

Supplemental Table 6: Summary of single gene manipulations of Calvin-Benson Cycle enzymes *in planta*.

Grape Cultivar	Carbon Dioxide Treatment	Yield Changes	Fruit Quality Changes	Reference
Cabernet Sauvignon	Ambient + 20 %	Up to 17.3 % increase in fruit yield	Average 1.7 % decrease in must sugar content, 13.6 % increase in total sugar harvested	(Wohlfahrt et al., 2018)
Riesling	Ambient + 20 %	Up to 17.8 % increase in fruit yield	Average 1.3 % decrease in must sugar content, 14.9 % increase in total sugar harvested	
Sangiovese	550 ppm	35 % – 42 %	2 – 5 % decrease in fruit acid content, 1 – 2 % increase in fruit sugar content.	(Bindi et al., 2001, 2005)
	700 ppm	37 % – 45 %	4 – 7 % decrease in fruit acid content, 1 – 2 % increase in fruit sugar content. Wine flavonoid content increased by average 35 %, anthocyanin content increased by average 23 %, colour intensity increased by average 41 %	

Supplemental Table 7: Summary of Free-Air Carbon Enrichment studies performed in grape.

Transgene Origin	Transgene Overexpressed	Plant	Biomass and Yield	Reference
Photores-piration	GCS-H	Arabidopsis	33 % increase in plant biomass for transgenic plants compared to WT at growth stage when inflorescence is first visible. Plants grown in controlled conditions.	(Timm et al., 2012)
			50 % increase in plant biomass for transgenic plants compared to WT at growth stage when inflorescence is first visible. 24% - 51% increase in biomass dependant on growth conditions at seed harvest. No observed increases in seed yield.	(Simkin et al., 2017)
		Tobacco	13 % – 38 % increase in above ground biomass of transgenic plants grown in field conditions.	(Lopez-Calcagno et al., 2018)
	GCS-L	Arabidopsis	19% – 47 % increase in biomass of transgenic plants grown in controlled environment conditions.	(Timm et al., 2015)
	GCS-T	Arabidopsis	No significant difference between transgenic lines and WT lines on any measure of plant biomass.	(Timm et al., 2018)
	SHMT	Poplar	Increase in biomass (25 % increase in diameter, 46 % increase in height) and up to 8.72 % increase in cell wall glucose.	(Zhang et al. 2019)
Photosynthetic Electron Transport Chain (ETC)	Cytochrome C_6	Arabidopsis	90 % increase in plant height and 40 % increase in root length at 40 days after planting. Flowering 7 – 12 days earlier in transgenic lines. Grown under controlled conditions.	(Chida et al., 2007)
		Tobacco	~70 % increase in plant height for most productive transgenic line versus WT.	(Yadav et al., 2018)

	Rieske Fe-S	Arabidopsis	29 % – 72 % increase in plant biomass at 25 days after planting. 31 % – 48 % increase in plant biomass at full maturity (52 days after planting). 51% increase in seed yield in the best line. Grown in controlled environment conditions.	(Simkin et al., 2017) (Zhang et al., 2020)
		Setaria viridis	> 40 % increase in total plant biomass at stage 5 of growth for 2 independent lines grown in controlled conditions. ~10% increase in complex components, increased photosynthetic efficiency, no reported increase in biomass.	(Ermakova et al., 2019)
	Ferrodoxin (Fd)	Tobacco	No increase in photosynthetic efficiency and no change to biomass yield was observed.	(Yamamoto et al., 2006)
	Ferrodoxin-NADP(H) reductase (FNR)	Tobacco	Up to 19 % reduction in aerial dry weight in transgenic lines at low light (150 µmol m^{-2} s^{-1}) and up to 12 % reduction in aerial dry weight in transgenic lines at high light (700 µmol m^{-2} s^{-1}). Plants grown in controlled conditions.	(Rodriguez et al., 2006)
	Plant ferrodoxin-like protein (PFLP)	Rice	Up to 47 % increase in panicle number per plant and up to 51 % increase in tiller number per plant. Up to 67 % increase in 1000-grain weight and up to 41 % increase in panicle weight. Induced increases in SBPase, FBPase, AGPase and sucrose-phosphate synthase (SPS) observed in these plants. Plants grown in glasshouse conditions.	(Chang et al., 2017)

Supplemental Table 8: Summary of single gene manipulations of photorespiration and photosynthetic electron transport chain enzymes *in planta*.

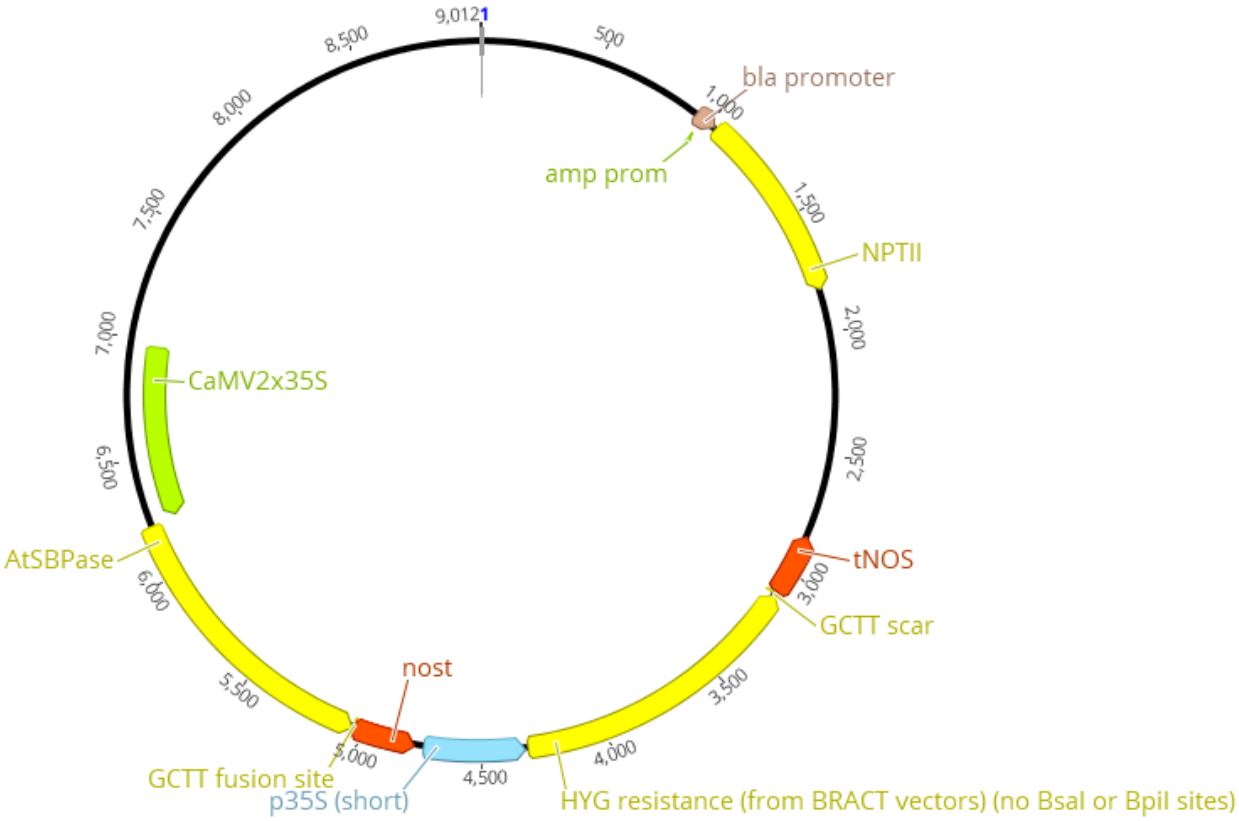
Transgene Origin	Transgene Overexpressed	Plant	Biomass and Yield	Reference
Bacterial – predicted carbon transport	ictB	Arabidopsis Rice Soybean Maize	> 25 % increase in dry weight of 18-day old transgenic plants relative to WT at low humidity (25 % – 30 %). Grown in growth chamber. 10 % – 120 % increase in panicle/tiller number per plant and 10 % – 70 % increase in grain number per plant for transgenic versus WT lines. Plants grown in field conditions. 15.1 % – 30.2 % increase in seed mass per plant and 12.7 % – 29.7 % increase in total plant mass at ambient [CO_2]. 23.1 % – 30.7 % in seed mass per plant and 27.8 % – 34.9 % increase in total plant mass at elevated [CO_2]. Data taken from results of two field seasons. 3.49 % increase in yield in transgenic plants versus WT, with up to a maximum 9.4 % increase in yield. Data collected over 2 years for summer and winter crops grown in field conditions.	(Lieman-Hurwitz et al., 2003) (Yang et al., 2008) (Hay et al., 2017) (Koester et al., 2021)
Plant membrane channels	Aquaporins	Rice Tobacco	14% increase in CO2 assimilation rate and a 27% increase in stomatal conductance (gs). 48% increase photosynthetic rate, 52% higher mesophyll conductance and enhanced growth under the well-watered growth conditions.	(Hanba et al. 2004) (Kawase et al. 2013).

Supplemental Table 9: Summary of single gene manipulations of genes associated with carbon transport *in planta*.

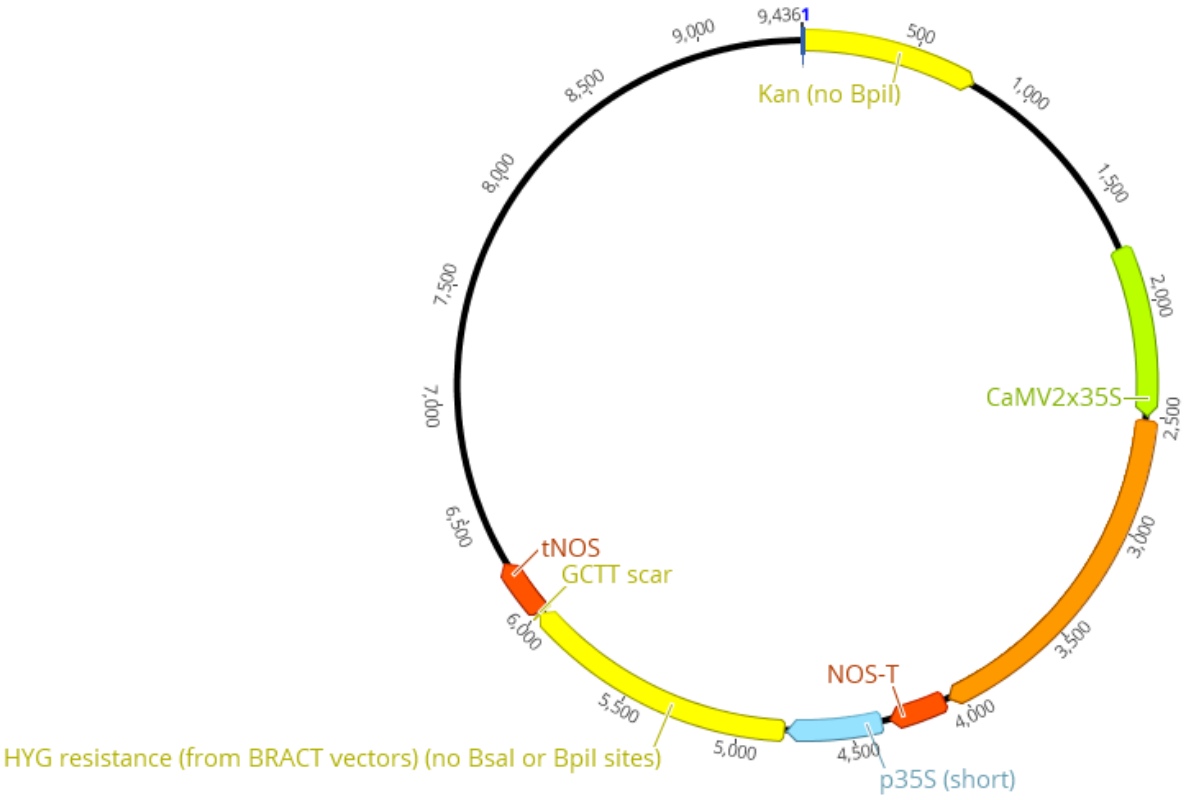
Sample	Increase in AtAGPase expression relative to FaAGPase expression (%)
5	170.5
6	162.4
7	269.4
8	232.6
9	207.9
13	146.3
14	172.6
15	85.2
20	148.6
24	93.7
45	183.0
47	155.6
WT	N/A

Supplemental Table 10: Increase in AGPase expression relative to endogenous AGPase. Change in expression calculated using the following formula:

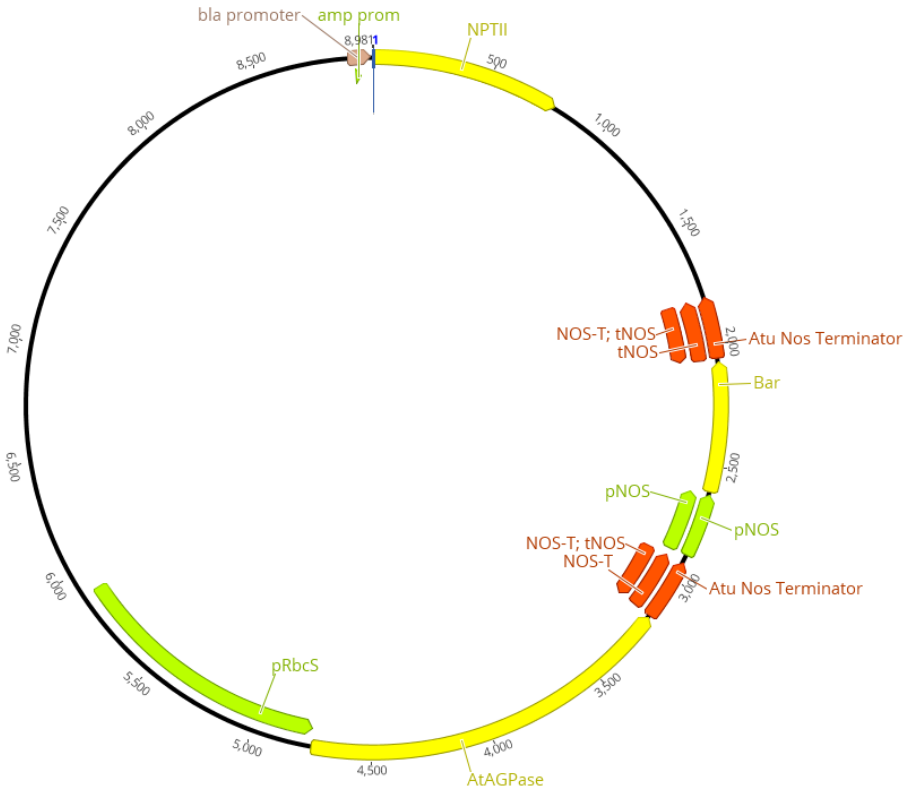
Percentage change = $2^{(\text{FaAGPase Expression} - \text{AtAGPase Expression})} \times 100$.



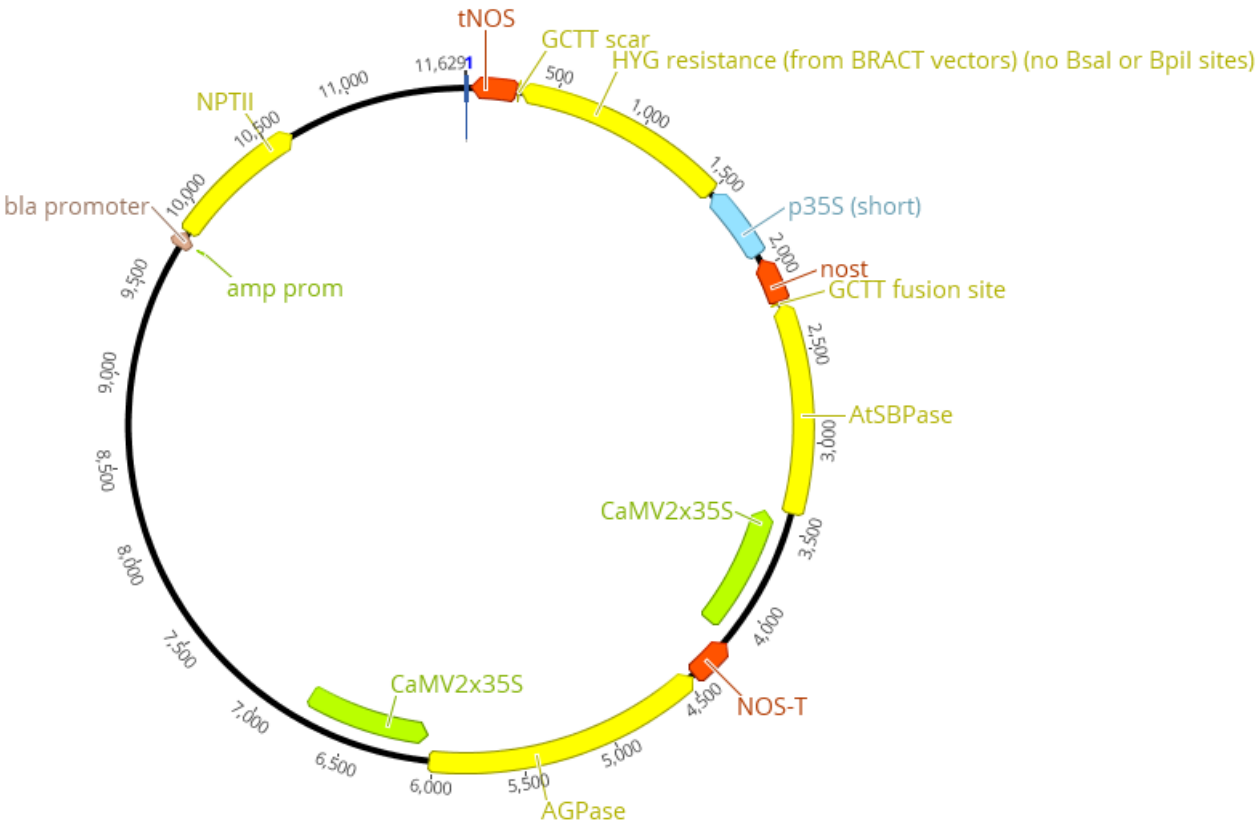
Supplementary Figure 1: Geneious map of Plasmid A for constitutive SBPase expression with hygromycin resistance for plant selection. Total construct length: 9012 bp. See 2.3.4 for method of construction and **Fig. 2.4.A** for schematic representation of plasmid.



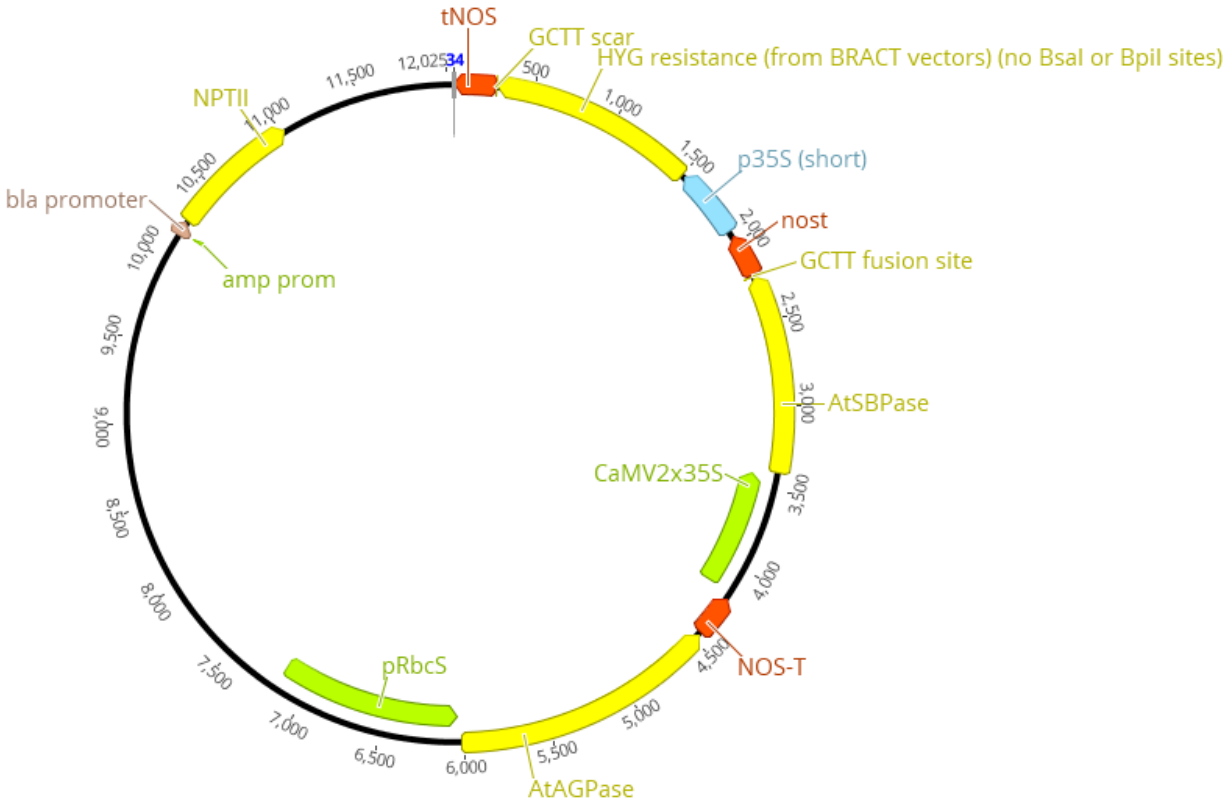
Supplementary Figure 2: Geneious map of Plasmid B for constitutive AGPase expression with hygromycin resistance for plant selection. Total construct length: 9436 bp. See 2.3.4 for method of construction and **Fig. 2.4.A** for schematic representation of plasmid.



Supplementary Figure 3: Geneious map of Plasmid C for green tissue specific AGPase expression with glufosinate resistance for plant selection. Total construct length: 8981 bp. See 2.3.4 for method of construction and **Fig. 2.4.D** for schematic representation of plasmid.



Supplementary Figure 4: Geneious map of Plasmid D for constitutive SBPase expression and constitutive AGPase expression with hygromycin resistance for plant selection. Total construct length: 11,629 bp. See 2.3.4 for method of construction and **Fig. 2.4.D** for schematic representation of plasmid.



Supplementary Figure 5: Geneious map of Plasmid E for constitutive SBPase expression and green tissue specific AGPase expression with hygromycin resistance for plant selection. Total construct length: 12,025 bp. See 2.3.4 for method of construction and **Fig. 2.4.E** for schematic representation of plasmid.