Final Report

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Title: Realising Increased Photosynthetic Efficiency to Increase Strawberry Yields

Short title for the website: Increasing Strawberry Photosynthesis

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Report No: [AHDB Use only]

This is the final report of a PhD project that ran from 10/2018 to 10/2022. The work was funded by AHDB as part of the CTP FCR (BBSRC and industry consortium headed by Berry Gardens).

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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Industry Summary

This project aims to improve strawberry fruit yield per hectare and strawberry fruit quality by increasing leaf photosynthetic performance through genetic manipulation of relevant enzymes.

As the global population continues to rise and climate change threatens current crop yields, new solutions are required to increase agricultural and horticultural productivity. Early studies have suggested that photosynthetic efficiency is a limiting factor on maximised crop growth and thus represents a target for improving yield. Previous work has demonstrated that genetically manipulating a key process of photosynthesis (the Calvin-Bensom Cycle, CBC) results in an increase in biomass yield and grain yield in tobacco and wheat respectively. Enhancing photosynthesis through genetic manipulation of this process is therefore demonstrative of a viable and effective method for improving photosynthetic efficiency across species. My work continues this line of research.

This work is being carried out in cultivated strawberry (*Fragaria x ananassa Duch.*), as current research in this area aims to understand how fundamental research in model plants, such as Tobacco and Arabidopsis, can be applied to crops. I will aim to determine if improvement of the CBC, through genetically manipulating one or multiple enzymes, results in changes to total biomass, harvestable yield, and developmental characteristics. Another key area of my research is to ascertain if enhanced photosynthesis, increased total biomass and increased fruit yield affect strawberry fruit quality. I will investigate this using a range of analytical chemistry techniques to study if or how key flavour and nutritional compounds differ between genetically modified and unmodified lines.

In the first year of this project, plasmids (loops of DNA) containing the CBC enzyme SBPase (sedoheptulose-1,7-bisphosphatase) and the starch synthesis enzyme AGPase (adenosine diphosphate glucose pyrophosphorylase) were constructed. Insertion of these plasmids (transformation) into strawberry leaves and subsequent regeneration of transformed plants from this tissue were tested in a range of cultivars. Regeneration was shown to be successful for the cultivar Calypso and the experimental line EMR 773. Successfully transformed and fully regenerated plants of Calypso were generated after extensive troubleshooting of the transformation method. EMR 773 was found to be recalcitrant to regeneration, though transformation of the tissue is achievable. Greater than 50 rooting transgenic Calypso lines were successfully generated with the double expressing construct and were weened onto soil for phenotyping. Analysis through chlorophyll fluorescence imaging revealed enhanced photosynthetic properties of transgenic lines.

Methods for extracting and analysing flavour components of strawberry fruit have also been developed in preparation for understanding how manipulating primary carbon metabolism in the leaf influences secondary carbon metabolism of strawberry fruit flavour. Results identified the high sensitivity of flavour compounds to different extraction methods, fruit age and cultivar type, highlighting the need for strict controls in this field of analysis.

Extensive field work has been carried out examining the influence of polytunnel row position on photosynthesis and yield of strawberry. Plants grown in the easternmost of the centre two rows (Row 4) displayed approximately 10 % - 20 % greater photosynthesis; this is in line with a historical 10 % - 15 % greater yield of Row 4 strawberries. Yield data has shown that, in the centre of the polytunnel, rows 3 and 4 yield approximately 17 % greater than rows 1 and 6. While row 4 was found to be the highest yielding row, row 3 had a similar yield (a difference of ~1.5 %). This segregation of yield between inner and outer rows may be indicative of marginally warmer temperatures generating more favourable growth conditions in the centre of a polytunnel. Historical yield differences between row 4 and all other polytunnel rows may derive from yield differences at polytunnel ends.

While it is difficult to ascertain exact financial payoffs at this stage of the project, it is reasonable to hypothesise that the project will have similar benefits on fruit growth and quality of carbon dioxide enrichment, as this also improves atmospheric carbon assimilation. This method, employed extensively by the Dutch horticultural industry, has been shown to increase strawberry fruit soluble sugar by up to 20 % (Wang and Bunce, 2004) and roughly double fruit dry weight production per plant (Sun et al., 2012). These data imply that genetic manipulation may also be capable of improving both fruit yield and quality, which could result in large monetary returns for growers.

Introduction

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. This issue is further compounded by rising global temperatures; meta-analysis of wheat productivity data has shown a worrying trend of decreased grain yield with increasing temperature (Asseng et al., 2015). While it has previously been postulated that this loss of yield may be offset by the simultaneous rise in atmospheric carbon dioxide (CO_2) (Parry et al., 2004), recent field trials of the C3 model plant soybean demonstrate that elevated atmospheric CO_2 is insufficient to rescue yield losses caused by drought conditions from increased temperature (Gray et al., 2016). In an effort to tackle this problem effectively, a large body of research has developed concerning improving carbon assimilation and photosynthesis to increase plant productivity and yield of both model and crop species (for comprehensive reviews see Simkin et al., 2019 and Weber and Bar-Even, 2019). It is hoped that this approach will create "future-proofed" plants, capable of feeding a growing populous while exploiting predicted environmental changes. This project will apply this approach to the horticultural crop strawberry (*Fragaria x ananassa. Duch*) by overexpressing rate limiting enzymes involved in carbon metabolism and photosynthesis to increase flux through desirable metabolic pathways and consequently increase yield (reviewed in Simkin et al. 2019). The total UK retail berry market was worth £1.27Bn for the year ending March 2018 (Kantar) with strawberry accounting for 47.4% of retail sales of UK berries. The berry market has grown by almost 33% (£400m) in the last 4 years and is projected to be worth £2Bn per annum by 2020. Increasing the yields and reducing the 'time to harvest' of strawberry crops will make a significant advance towards maintaining global and UK food resources for the 21st century as well as protecting diverse environments within the UK and Europe.

Crop yield can be defined by the yield equations:

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

$$Y_p = \eta * P_n$$

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 from Morgan et al., 2005 and Dermody et al., 2008 on field grown soybean

was analysed to calculate the harvest index, conversion efficiency and interception efficiency of the crop. Both harvest index and interception efficiency were found to be near their theoretical maxima of ~ 0.9 and ~ 0.6, however conversion efficiency was ~ 30 % of its theoretical maximum of ~ 0.1. This reveals how conventional breeding has struggled to achieve the theoretical maximum conversion efficiency and therefore carbon assimilation is a major limitation on crop yield. This leaves genetic modification as the best approach to improve on this major barrier to maximised yields.

While several approaches exist to improve photosynthetic efficiency, this project focusses on overexpressing rate-limiting enzymes of the Calvin-Benson Cycle (CBC) and other enzymes that also improve photosynthetic parameters when overexpressed. This method has been demonstrated to work across various model and crop species for the CBC enzymes SBPase (sedoheptulose-1,7-bisphosphatase, EC 3.1.3.37, Ding et al., 2016; Driever et al., 2017; Lefebvre et al., 2005; Rosenthal et al., 2011) and FBPA (fructose bisphosphate aldolase, EC 4.1.2.13, Uematsu et al., 2012). Manipulation of photorespiration has also been shown to improve photosynthetic efficiency (Lopez-Calcagno et al., 2018; Timm et al., 2012, 2015), as has manipulation of photosynthetic electron transport (Chang et al., 2017; Chida et al., 2007; Ermakova et al., 2019; Simkin et al., 2017b; Yadav et al., 2018) and the expression of the bacterial carbon transporter ICTB (Hay et al., 2017; Lieman-Hurwitz et al., 2003, 2005).

Manipulation of several of these targets simultaneously was found to further enhance photosynthesis and growth more than manipulating a single target (Gong et al., 2015; Simkin et al., 2015, 2017a). in one study, in which SBPase, FBPA and ICTB were simultaneously overexpressed in the model plant Tobacco, plant dry weight more than doubled (103 % increase) compared to wild type in the line overexpressing all 3 genes. Plants overexpressing just SBPase or ICTB had dry weight increases compared to wild type of 34 % and 71 % respectively – still impressive gains, but less than that achieved by manipulation of multiple targets (Simkin et al., 2015). As such, multigene manipulation is being employed in this project in an effort to maximise photosynthetic efficiency and productivity.

2 major challenges are presented in applying this work to strawberry: sink limited growth of fruit and the impact of manipulating photosynthesis on fruit flavour and quality.

Work by Hansen, P. 1989 compared fruit development of control plants and plants which had half of their flowers removed. Unsurprisingly, reduction in flower number reduced the number of fruits. More interestingly however, this did not correlate with an increase in the size of fruits (Hansen, 1989). With a reduced number of sink tissues (i.e. fruits) available, if strawberry were source-limited these sink tissues would grow larger. This is not the case, providing evidence that strawberry fruit growth is a sink limited process. Since this project works on

improving source capacity by increase photosynthetic carbon assimilation, it is important that sink capacity is also manipulated for maximum benefit of source manipulations to be realised. While several approaches exist to manipulate source-sink allocation by genetic engineering (see Sonnewald and Fernie, 2018 for a current opinion piece on the subject), the most extensively studied target is AGPase, the enzyme that irreversibly commits Calvin Cycle products to starch synthesis and catalyses the following reaction:

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

where ATP is adenosine triphosphate, ADPglucose is adenosine diphosphate glucose and PP_i is pyrophosphate (Tuncel and Okita, 2013). This is a particularly interesting target for this project, since Zhu et al., 2007 predicted that AGPase was needed in higher concentrations in photosynthetic tissue for maximum photosynthetic efficiency. As such, AGPase overexpression could simultaneously improve source-sink relations to ensure biomass is allocated to the growing sink tissue and improve photosynthetic efficiency.

Strawberry flavour is highly complex, with reviews reporting over 300 reported volatiles across the current literature (Pérez and Sanz, 2010; Yan et al., 2018; Zabetakis and Holden, 1997). Further studies report that these volatiles do not contribute equally to the flavour composition of fruits and that variance in volatile composition is dependent on a range of environmental factors, cultivar type and post-harvest treatments (Forney et al., 2000). Due to volatile biosynthesis being highly sensitive to even small changes, it is hypothesised that manipulation of primary carbon metabolism will affect the volatile composition of transgenic fruit compared to WT cultivars. In addition, strawberry flavour is strongly influenced by sugar and organic acid concentrations and the presence or absence of different amino acids and phenolic compounds, adding an important non-volatile dimension that may also be influenced by transformations.

It is clear that increased photosynthetic efficiency has great potential for improved plant growth and that transgenic approaches are a viable method of achieving this. Applying this to strawberry will have implications on carbon allocation, flavour and harvest time in addition to changes in growth and this project will account for these. From considering the literature, it was decided that transformations with SBPase and AGPase would be studied first as together these genes appear to have the greatest potential for improving strawberry yields.

Materials and methods

Strawberry micropropagation and growth conditions

Stocks of cultivated strawberry (*Fragaria x ananassa Duch.*, cv. Calypso, EMR-773, EMR-2434-1) 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 Appendix 1 for media recipes.

cDNA synthesis

Arabidopsis thaliana was grown at 120 µmol m^{-2} s^{-1} with a 16/8 photoperiod. RNA was extracted from young developing leaves as directed by Macherey-Nagel NuceloSpin RNA kit. Forward SBPase primer: CACCATGGAGACCAGCATCGCGTGC. Reverse SBPase primer: GTTTCTAAGCGGTAACTCCAATGG. Forward AGPase primer: CACCATGGTGGTCTCTGCTGACTGC. Reverse AGPase primer: CTTAAAAGTATCATATCACAACTCC. Synthesis and subsequent amplification of cDNAs was performed as directed by Invitrogen SuperScript III Reverse Transcriptase kit. Samples were purified as directed by Macherey-Nagel NucleoSpin Gel and PCR Clean-up kit. All results were confirmed via gel electrophoresis using an agarose gel (0.75 %, TAE buffer) stained with gel red (10 µL L^{-1}).

Cloning

Purified cDNAs were cloned into the pENTR/D-TOPO vector as directed by Invitrogen pENTR/D-TOPO Cloning Kit (see supplementary Figure 1 for vector map).

Chemical transformation of Escherichia coli

Stock of *E. coli* (strain DH5α) stored in glycerol (25 %) 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) was added to cells. Cells plated on high salt LB plates with kanamycin (50 µg mL^{-1}) and incubated at 37 °C overnight. Successful transformation was confirmed using colony PCR (see below).

Colony PCR

Individual colonies of *E. coli* were collected with a sterile pipette tip and touched against a high salt LB plate with kanamycin (50 µg mL^{-1}) before being added to a PCR mix (see master mix recipe in Appendix 2). Plate was incubated at 37 °C overnight, with successful colony

growth indicating successful transformation. PCR was ran as follows: 95 °C for 2 minutes; 45 cycles of 95 °C for 30 seconds, followed by 55 °C for 30 seconds, followed by 72 °C for 1 minute 45 seconds; 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 %, TAE buffer) stained with gel red (10 µL L^{-1}).

Golden Gate assembly

Level 0 genetic elements and Level 1 destination vectors donated by the University of Essex (see plasmid maps in **Supplementary Figure 2**). Successful cloning was confirmed by white-orange selection of transformed *E. coli* (see above protocol) and gel electrophoresis on agarose gel (0.75 %, TAE buffer) stained with GelGreen.

Sequencing

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

Chemical transformation of Agrobacterium tumefaciens

Stock of *A. tumefaciens* (strain EHA105) stored in glycerol (25 %) 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) 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). Bacterial suspension was plated on low salt agar plates with kanamycin (50 µg mL^{-1}) and incubated at 28 °C for 48 hours. Plates exhibiting successful colony growth were sealed with parafilm and stored at 4 °C.

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) with kanamycin (50 µg mL^{-1}) and rifampicin (25 µg mL^{-1}). Samples were shaken at 28 °C overnight. Starter culture (1 mL) was added to low salt LB broth (20 mL) with kanamycin (50 µg mL^{-1}) and rifampicin (25 µg mL^{-1}) 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}) supplemented with glucose (30 g L^{-1}) and acetosyringone (100 µM) 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 subbed to fresh media without selection and stored in light under ~68 µmol m^{-2} s^{-1} light intensity with a 16/8 photoperiod for a week. Explants were then transferred to SRM plates containing hygromycin (25 µg mL^{-1}) or bialaphos (5 µg mL^{-1}) (depending on the selectable marker present in the plasmid backbone) and TCA (400 µg mL^{-1}). Plates were sealed with Parafilm 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. Media was changed every 4 weeks to account for hormone degradation.

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. Callus visible after approximately 2 months. Explants were excised from callus as appropriate and moved onto Fragaria Regeneration Medium (FRAG-R) to encourage root induction. Fully regenerated plants were transferred to SPM without selectable markers.

Strawberry sugars and acids extraction

Newly bought strawberries (n = 5) and 2 day old strawberries stored at 4 °C (n=5) were hulled and sliced thinly (cv. Eve's Delight, Sweet Eve). Chopped strawberries were placed in sealed bags and freeze-dried over several days. Freeze-dried samples were milled to a powder. Fresh samples (n = 5 berries) were blended in 4 parts water by weight. Freeze-dried strawberry powder (0.2 g) or fresh blended strawberry (0.2 g) was added to hydrochloric acid solution (10 mL, 0.01 M) and the mix stirred for 30 minutes at RTP. An aliquot of mixture (1.5 mL) was taken and centrifuged at maximum speed for 30 minutes. Samples were filtered through a 0.22 µm filter and stored at 4 °C.

Strawberry phenolic compounds extraction

Freeze-dried strawberry (50 mg, cv. Eve's Delight, Sweet Eve, see "Strawberry sugars and acids extraction" for freeze-drying protocol) was added to aqueous methanol (1.5 mL, 90 % v/v) on ice and vortexed for 10 seconds. Samples were sonicated at ambient temperature for 20 minutes then centrifuged at maximum speed for 20 minutes at 4 °C. Supernatant was filtered through a 0.22 µm filter and samples stored at 4 °C.

Strawberry volatiles extraction

Fresh strawberries (n = 5, cv. Eve's Delight, Sweet Eve) were blended. Blended strawberry (0.2 g) or freeze-dried strawberry (0.2 g, see "Strawberry sugars and acids extraction" for freeze-drying protocol) were added to calcium chloride solution (2 mL, saturated) on the day of processing.

Chemical analysis

Extracted compounds were analysed by HPLC (sugars, acids), UPLC (sugars, phenolics) or GC-MS (volatiles) using generic protocols.

RNA extraction of strawberry

Fully expanded non-senescent leaves of strawberry were taken after subbing for varieties Calypso and EMR-773. RNA was extracted using the Machery-Nagel NucleoSpin RNA Plant and Fungi Kit for PCR and qPCR and with the Thermofisher RNAqueous Kit with Plant Isolation Aid for RNAseq.

Plant growth conditions (field)

Rows of strawberries were planted in coir (Cocogreen) and arranged into elevated rows in a polytunnel as shown below in Figure A:

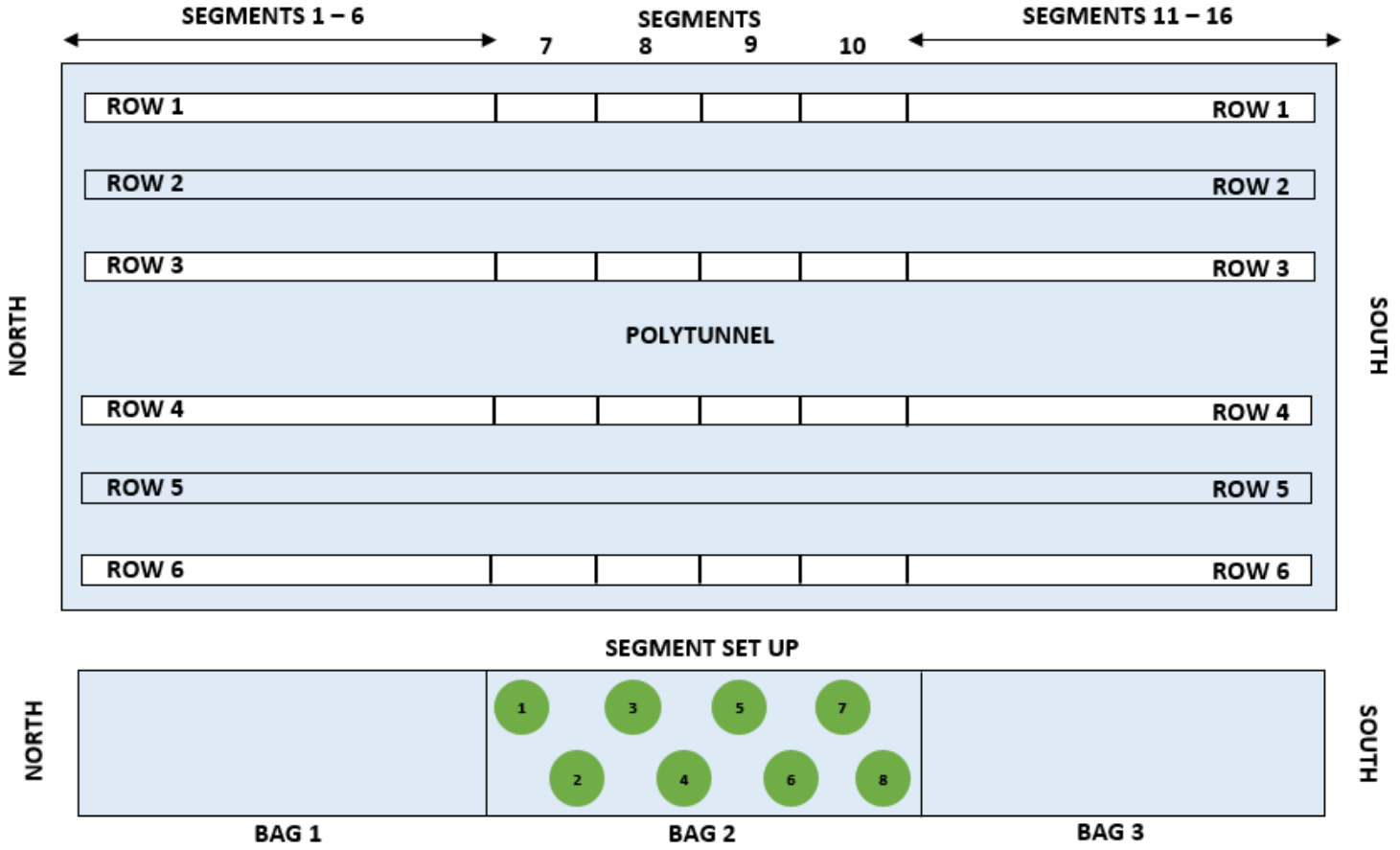

Figure A: Polytunnel setup for experimental work. Rows highlighted in white were studied. Plants were planted as follows: 8 plants per bag, 3 bags per segment and 16 segments per row.

Photosynthetic data collection (field)

Measurements were taken using the LCpro-SD iFL Portable Photosynthesis System (ADC). Point measurements were taken at saturating light (1500 µmol m^{-2} s^{-1}) with no other environmental conditions controlled. Light curves were taken using the following programme of steps with measurements taken at one minute intervals:

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

Fruit harvesting (field)

Ripe fruits were harvested twice weekly. Class 1, Class 2 and waste yields were recorded. 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.

Leaf harvesting (field)

Leaves tested during photosynthetic light curve measurements were harvested by cutting into 3 smaller segments, during which the midrib was removed, and immersed immediately in liquid nitrogen.

Results

Initial cloning efforts failed to produce the desired product.

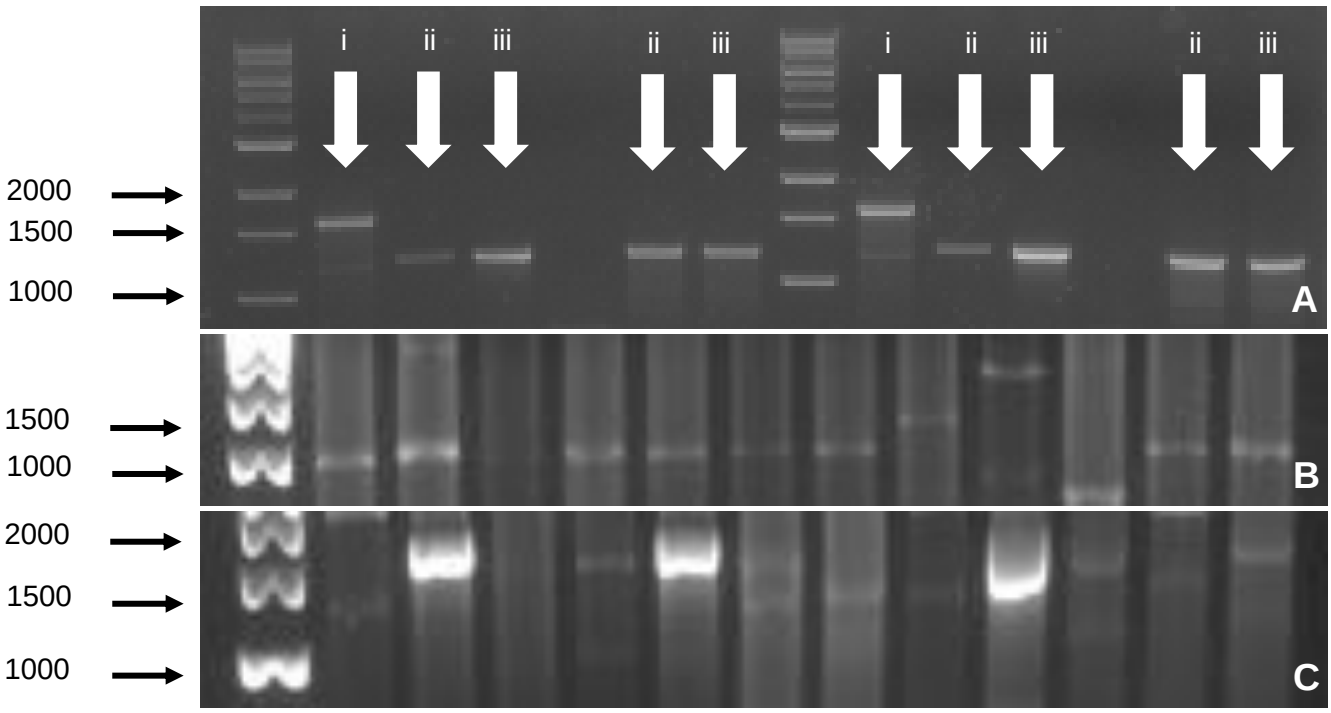

Figure 1: Gel electrophoresis scans showing progression of cloning experiments. **A:** Purified cDNAs obtained from reverse transcription of target gene mRNA; **i:** AGPase; **ii:** SBPase; **iii:** SBPase positive control. **B:** SBPase CDS fragments amplified from transformed *E. coli*. **C:** AGPase CDS fragments amplified from transformed *E. coli.*

RNA was extracted from aerial tissue of *A. thaliana* and cDNAs of SBPase and AGPase generated via reverse transcription. SBPase cDNA was 1182 nt and AGPase cDNA was 1596 nt. cDNAs were amplified and purified with this result being quantified via gel electrophoresis (**Figure 1A**). Following cloning of these fragments into a construct, *E. coli* were transformed with the construct and colony PCR was used to confirm construct insertion (**Figures 1B and C**). Smearing on these latter 2 gels was attributed to overloading the gel with bacterial DNA. However, due to several issues with this approach (see discussion), Golden Gate cloning was adopted instead.

Cloning via Golden Gate Assembly successfully generated desired constructs

Single gene constructs containing AtSBPase and AtAGPase coding sequences (CDS(s)) were donated by the University of Essex. The former constructs (**Supplementary Figure 2A and B**) contain glufosinate and hygromycin resistance genes for plant selection respectively with the SBPase CDS downstream of the cauliflower mosaic virus 35S (CaMV 35S) promoter in both cases. The latter constructs (**Supplementary Figure 2C and D**) contain the AGPase CDS downstream of the 35S promoter and RuBisCO small subunit promoter (RbcS) respectively. Collectively, this suite of constructs enables 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 (**Supplementary Figure 2E**) or RbcS (**Supplementary Figure 2F**) promoter were generated through Golden Gate assembly. Successful cloning of these constructs was confirmed by gel electrophoresis and sequencing.

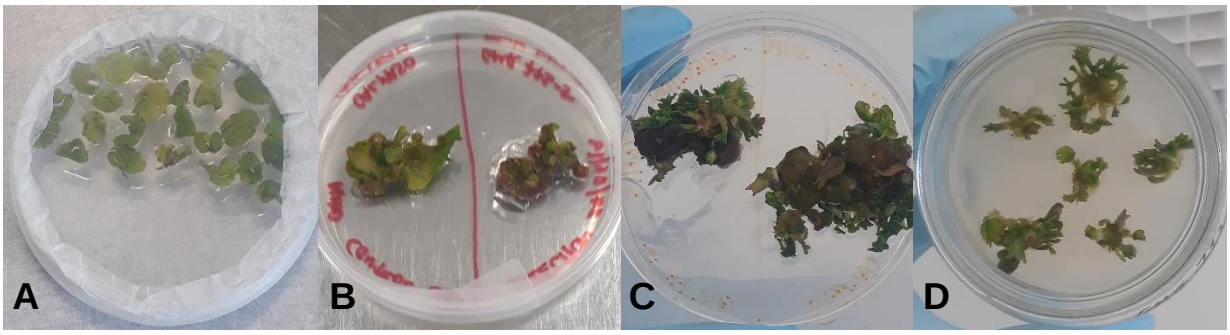

Figure 2: Timeline of strawberry regeneration from leaflet explants to separated strawberry shoots. **A:** Scored leaflet explants from 4 week old strawberry plants (Calypso cultivar shown) on shoot regeneration medium (SRM). **B:** Explants at 4 weeks of growth on SRM (Calypso left, EMR 773 right). **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).

Strawberry varieties Calypso and EMR 773-5 were successfully regenerated from leaflet explants

Regeneration of leaflet explants of Calypso and EMR 773-5 cultivars was demonstrated through growth on SRM without selectable markers over 12 weeks (see **Figure 2**). Callus induction was visible after 2 weeks on medium (not shown) with differentiated leaf and root tissue visible after 12 weeks of growth. Callus was divided to separate developing plantlets and moved to SPM. Phenotypically normal plant differentiation was visible 2 weeks after

division and transfer to SPM. Transformed leaflet explants and the experimental line 2434-1 developed to the callus stage but no further leaf development occurred.

Transformed plantlets of Calypso successfully regenerated from leaflet explants

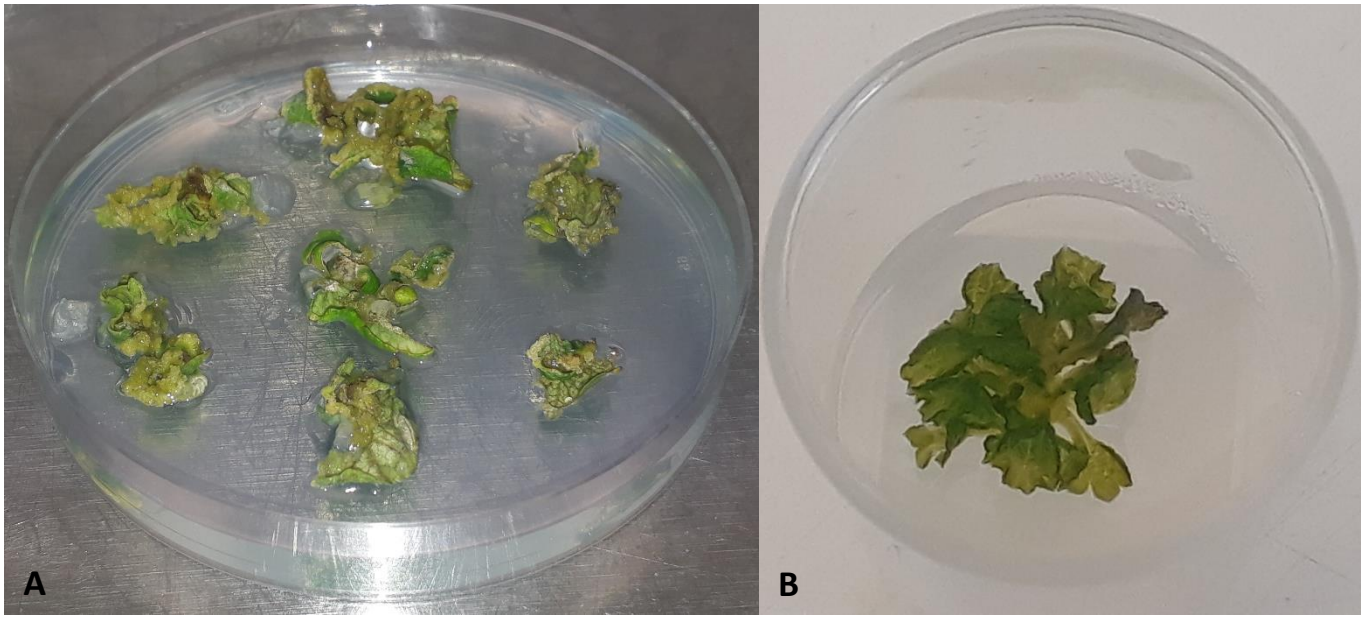

Figure 3: Timeline of strawberry regeneration of transformed callus from leaflet explants to separated strawberry shoots. **A:** Scored leaflet explants at 12 weeks old on shoot regeneration medium (SRM) supplemented with hygromycin. **B:** Transgenic strawberry shoot on Fragaria rooting medium (FRAG-R).

Strawberry leaflets transformed with plasmid F were grown on hygromycin selection (See **Figure 3**). Callus development was observed 8 weeks after transformation. Initial plantlet development was observed 5 months after transformation. 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. 50 independent rooting strawberry lines expressing the double plasmid were generated to be taken forward for future phenotyping. Genotyping of these lines revealed one line showing overexpression of only AGPase. This gave rise to three genetic lines for study: wild type (WT), AGPase overexpressing (A) and SBPase/AGPase co-overexpressing (SA).

Transgenic strawberry plants exhibited enhanced non-photochemical quenching associated with increased yield

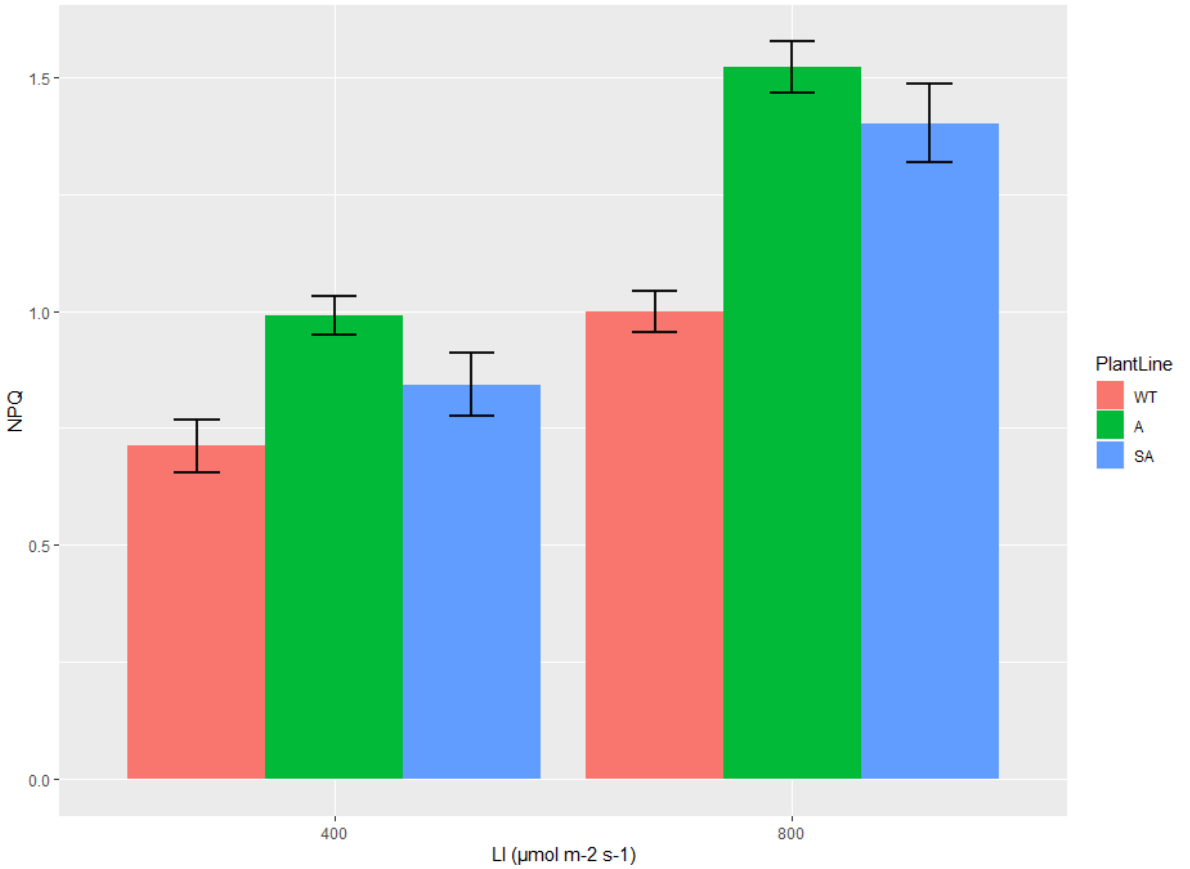

Fig. 11: NPQ of wild type (WT), AGPase overexpressing (A) and SBPase/AGPase co-overexpressing (SA) lines in response to actinic light at two given intensities.

Increased non-photochemical quenching (NPQ) was identified in both the AGPase overexpressing line and the SBPase/AGPase co-overexpressing line relative to the wild type. This indicated that transformation of the plants with these genes was sufficient to positively influence their photosynthetic traits, since elevated NPQ is associated with increased yield in strawberry.

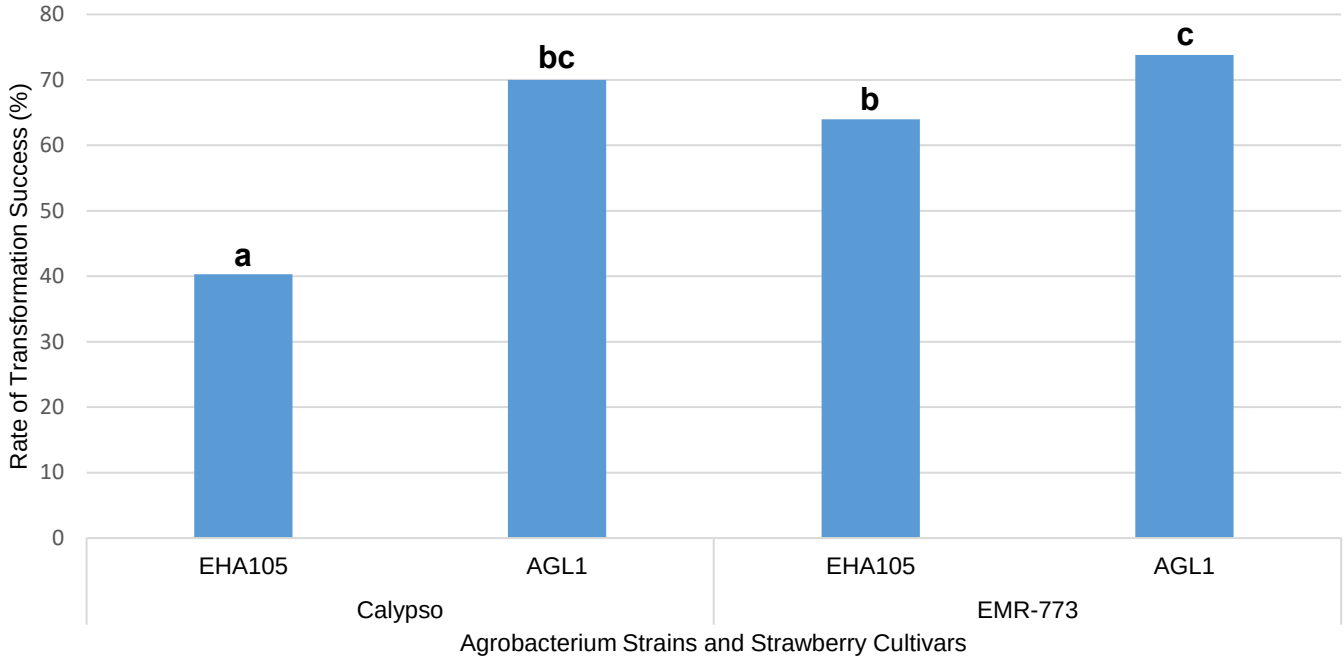

Figure 4: Comparison of transformation efficiency of Agrobacterium strains EHA105 and AGL1 for strawberry cultivars Calypso and EMR-773. Different letters above bars represent statistically significantly different results ($p < 0.05$).

Extensive troubleshooting has been performed to improve the strawberry transformation protocol. Callus induction was only achieved once a week long light exposure step following dark incubation was introduced to the protocol. In addition, only the cultivar Calypso has so far shown successful explant regeneration. 2 different Agrobacterium strains have also been tested for their capacity to successfully transfect Calypso and EMR-773 (see **Figure 4**), with AGL1 appearing to have greater capacity to transfect strawberry leaflets than EHA105, though transfection is achievable with reasonably high efficiencies with both bacterial strains.

Fructose is the predominant sugar in strawberry fruit and fruit glucose concentration increases with storage time

UPLC analysis was performed on the late everbearer variety “Eve’s Delight” to determine fruit concentration of 3 major sugars – glucose, fructose and sucrose. Significant differences in the concentration of these sugars were found, with fructose being the most abundant and sucrose the least (**Figure 5A**). Analysis of freshly bought fruits and strawberries kept in cold storage for 2 days identified a significant increase in fruit glucose concentration with storage time (**Figure 5B**), however no significant difference in sucrose or fructose concentrations could be determined (**Figure 5C and D**).

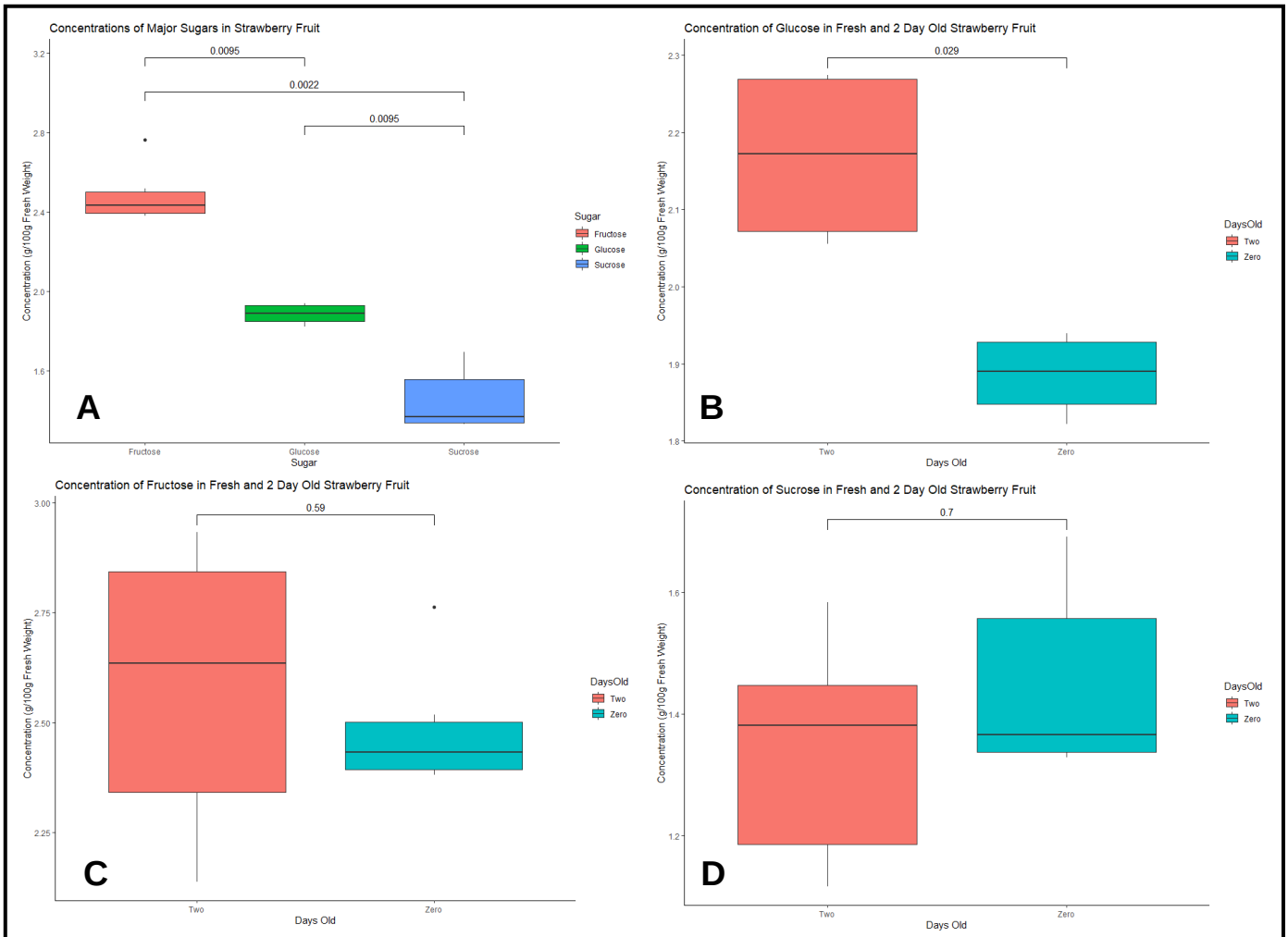

Figure 5: Box plots showing sugar concentration data for cv. Eve’s Delight. n = 4 – 6. **A:** Concentrations of the 3 major sugars in strawberry. **B:** Concentration of glucose in freshly bought and 2 day old strawberries. **C:** Concentration of fructose in freshly bought and 2 day old strawberries. **D:** Concentration of sucrose in freshly bought and 2 day old strawberries.

Strawberry fruit contains a significant repertoire of phenolic compounds

Screen Hits			Near Misses		
Compound Name	**Molecular Weight**	**Intensity**	**Compound Name**	**Molecular Weight**	**Intensity**
Catechin	288.9	1122264	Procyanidin B2	577	985144
Biochainin A Methyl Ether	297	162101	Luteolin-7-glucoside	447	762130
Biochainin A	282.9	22680	4-hydroxybenzoic acid	137.3	58951
Caffeine	194.9	18453	Quercitin neg	300.9	57301
2,4-dihydroxybenzoic acid	153	16497	Neochlorogenic acid	354.3	15499
Formononetin	266.9	16006			
4-CQA	353.1	15499			
Narigin	579.1	11724			
Diosmin	607.1	11203			

Figure 6: Table showing compounds identified in strawberry (“Screen Hits”) and compounds closely related to unidentified compounds in the screen (“Near Misses”). Cv. Sweet Eve.

A general phenolic compound screen via UPLC identified catechin and biochainin A methyl ether as abundant phenolic compounds in strawberry fruit. Procyanidin B2 and luteolin-7-glucoside were identified as highly related compounds to other, currently unidentified, abundant phenolic compounds. Proanthocyanidins, a oligermeric compound group predmoninatly formed of catechins, have been identified as a major strawberry volatile group (Buendía et al., 2010). This may explain the presence of catechin as an identified volatile and why procyanidin B2 was identified as a near miss. A full list of identified compounds can be found in **Figure 6**. Unfortunately, due to the lack of a standard to compare intensity, the concentration of these phenolic compounds cannot yet be determined.

Freeze-drying and cultivar type affect volatile repertoire of strawberry fruits

Volatile flavour components of fruits of fresh and freeze-dried cultivars “Sweet Eve” and “Eve’s Delight” were analysed via GC-MS. Unique and shared aromatic compounds were counted and the distribution between the different varieties and treatments is shown in **Figure 7**. A number of unique compounds, primarily formed of methyl, butyl and octyl esters, were identified in the premium “Eve’s Delight” cultivar compared to “Sweet Eve”, however these were lost after freeze-drying. Additionally, the premium variety contained mesifurane, a rare furanone that has been identified as being important for strawberry flavour (Ulrich et al.,

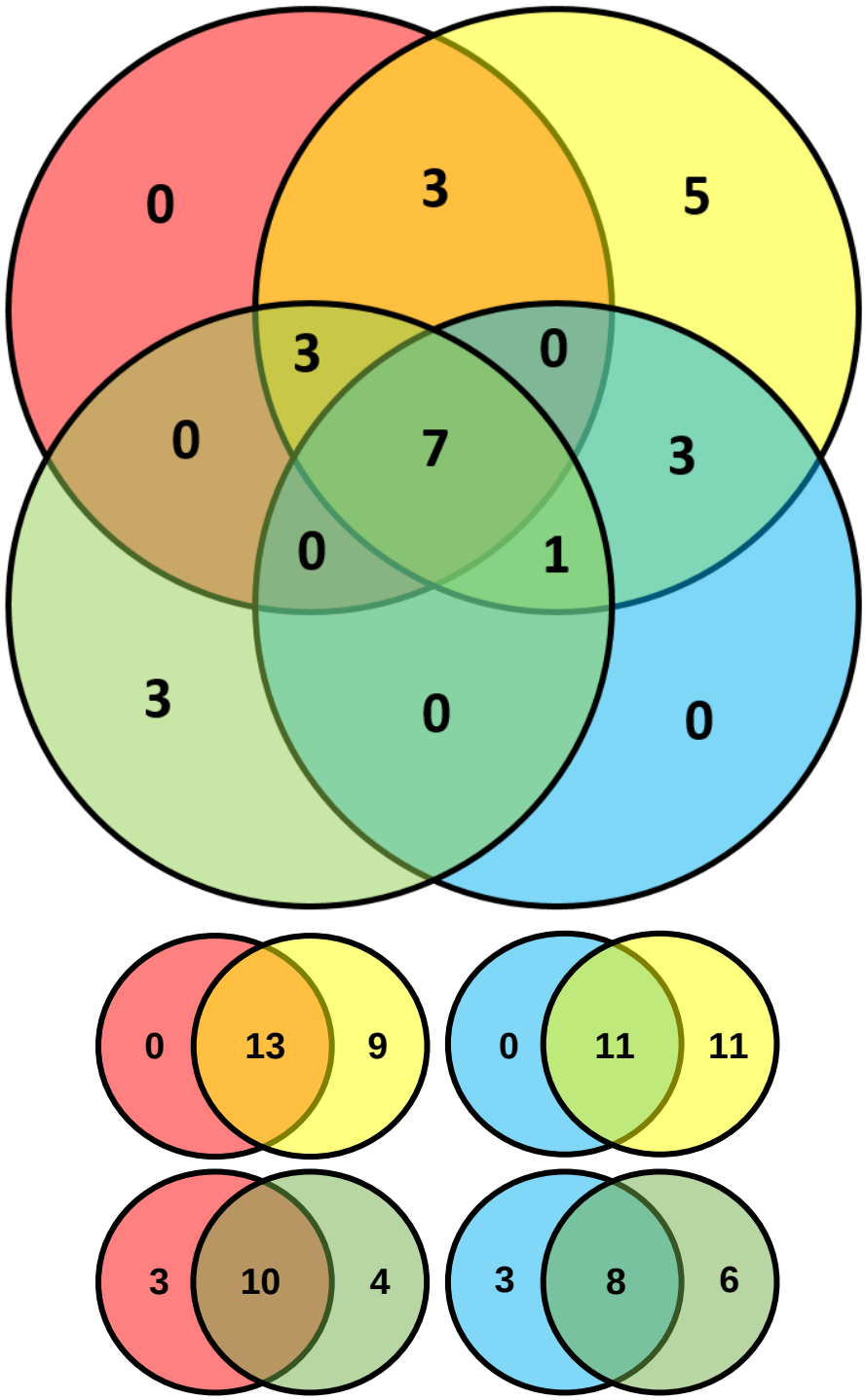

Figure 7: Venn diagrams showing the number of unique volatile compounds present in greater than trace quantities in cultivated strawberry. Compounds’ LRIs quantified against a NIST database. 5 berries per sample, n= 3. **Red:** Fresh strawberry, cv. Sweet Eve. **Yellow:** Fresh strawberry, cv. Eve’s Delight. **Green:** Freeze-dried strawberry, cv. Sweet Eve. **Blue:** Freeze-dried strawberry, cv. Eve’s Delight.

1997). 7 compounds were identified as being shared between all fruits tested; primarily esters and the important terpene linalool. For a full list of volatiles identified, see **Appendix 3**.

Row 4 strawberries have greater carbon assimilation during the morning with no significant difference found during the afternoon

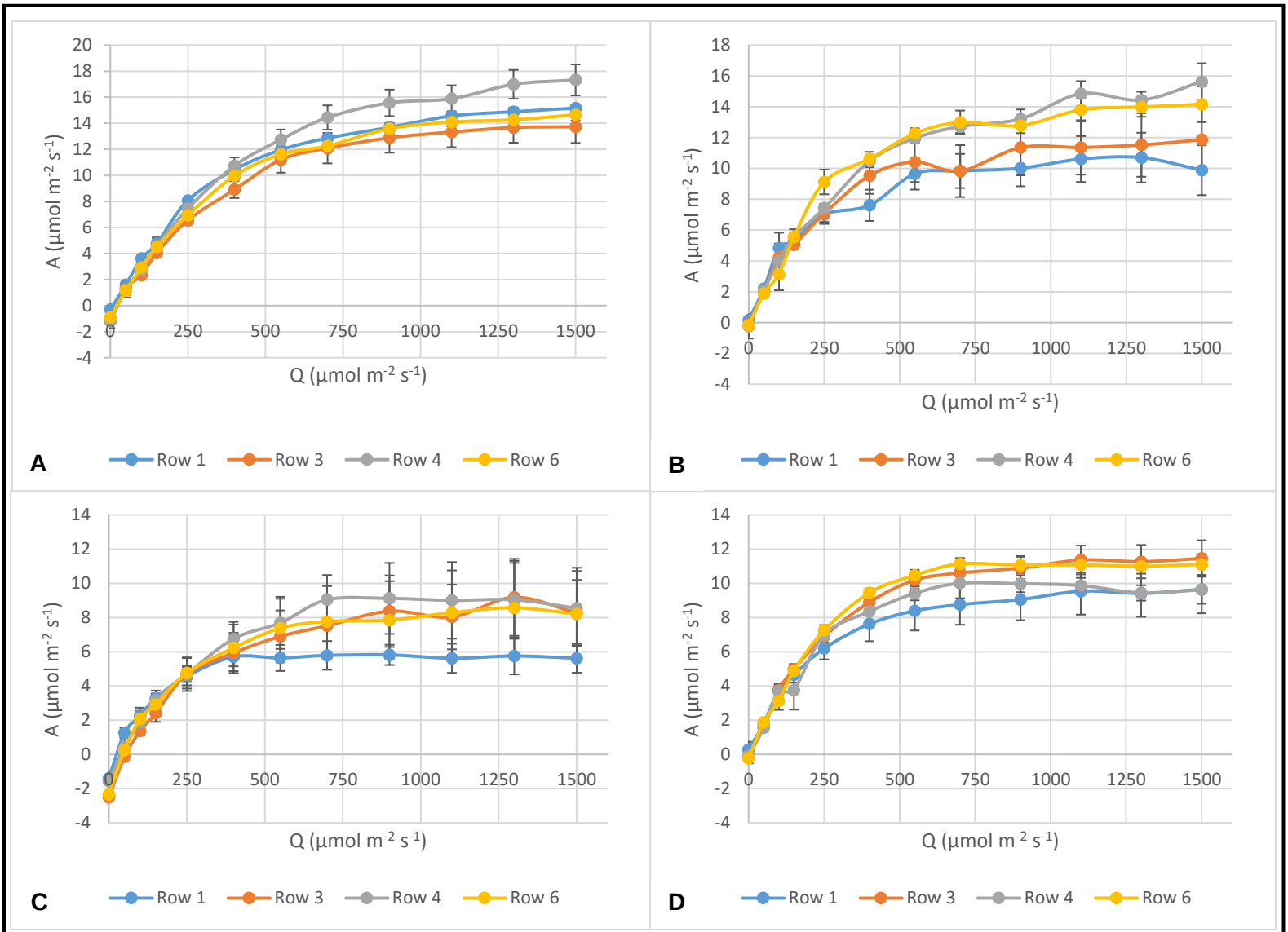

Figure 8: Light response curves of strawberry plants in different polytunnel rows during the early and mid season. A = rate of carbon assimilation; Q = light intensity. **A:** Early season morning light response curves. **B:** Mid-season morning light response curves. **C:** Early season afternoon light response curves. **D:** Mid-season afternoon light response curves. n = 4 – 6.

Light response curves of strawberry plants were taken to assess photosynthetic performance of plants across a polytunnel. These measurements were taken at two time points across the season to compare how photosynthesis may fluctuate as the plants age. Row 4 was found to have ~ 2 % - 31 % greater assimilation than any other row during the morning, regardless of time during the season. Row 4 also exhibited ~ 9 % - 42 % greater photosynthesis than any

other row in the afternoon of the early season, however rows 3 and 6 showed greater carbon assimilation in the afternoon than row 4 in the mid-season.

Yield of poltunnel grown strawberries segregates symmetrically across a tunnel

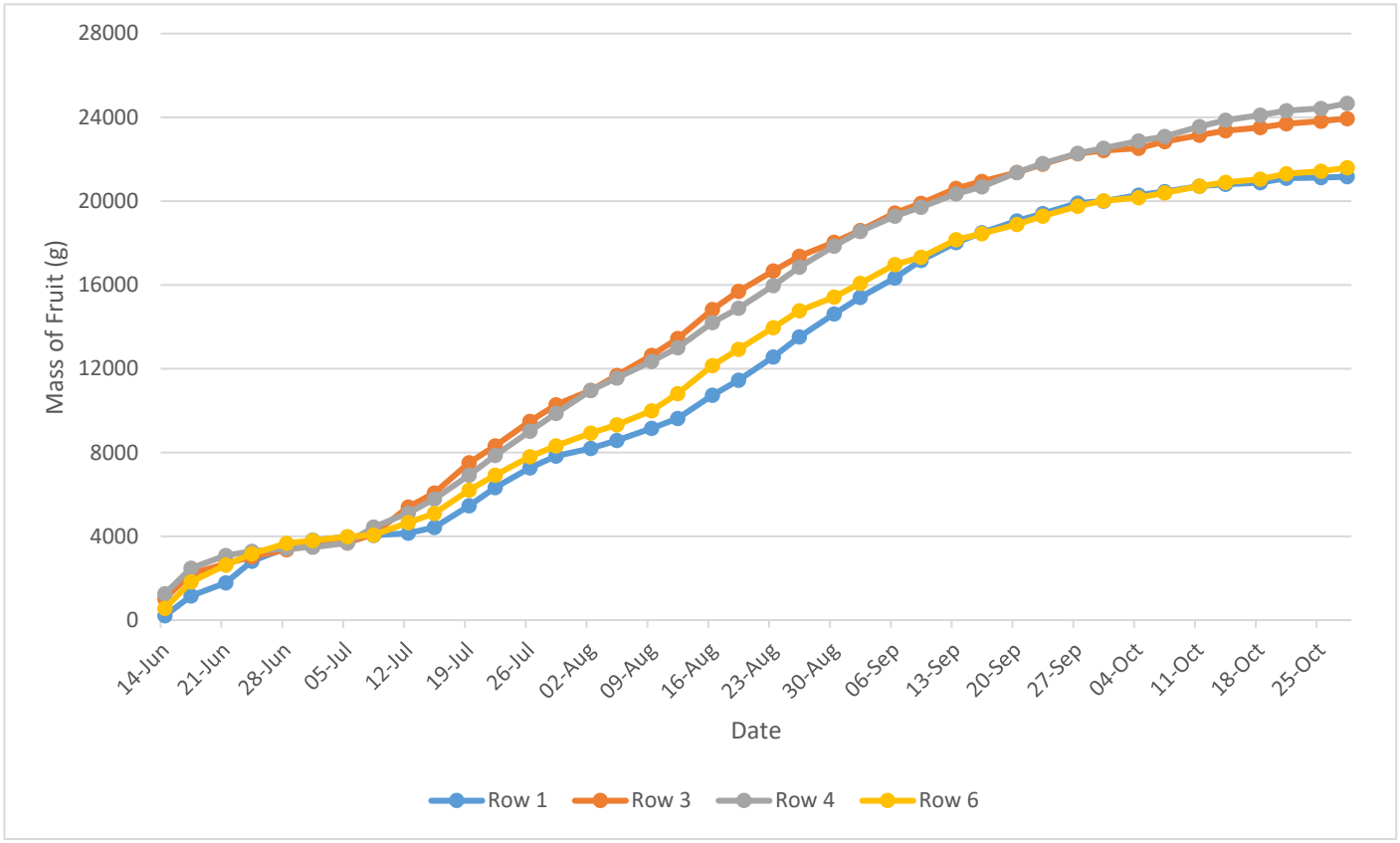

Figure 9: Cumulative Class 1 fresh fruit yields of strawberry fruit grown in different polytunnel rows.

Yield and number of Class 1, Class 2 and waste fruits of each polytunnel row were recorded. Row 4 showed the highest class 1 yield with ~ 2 % - 17 % greater yield in row 4 than in other polytunnel rows. This was derived from an increase in fruit number; row 4 produced ~ 6 % - 23 % more individual Class 1 fruits than any other row. Little difference between proportion of Class 1, Class 2 and waste fruits was found between rows (~81 % - 86 % Class 1 fruits by fresh yield).

East and West facing plants yield differently within a polytunnel row

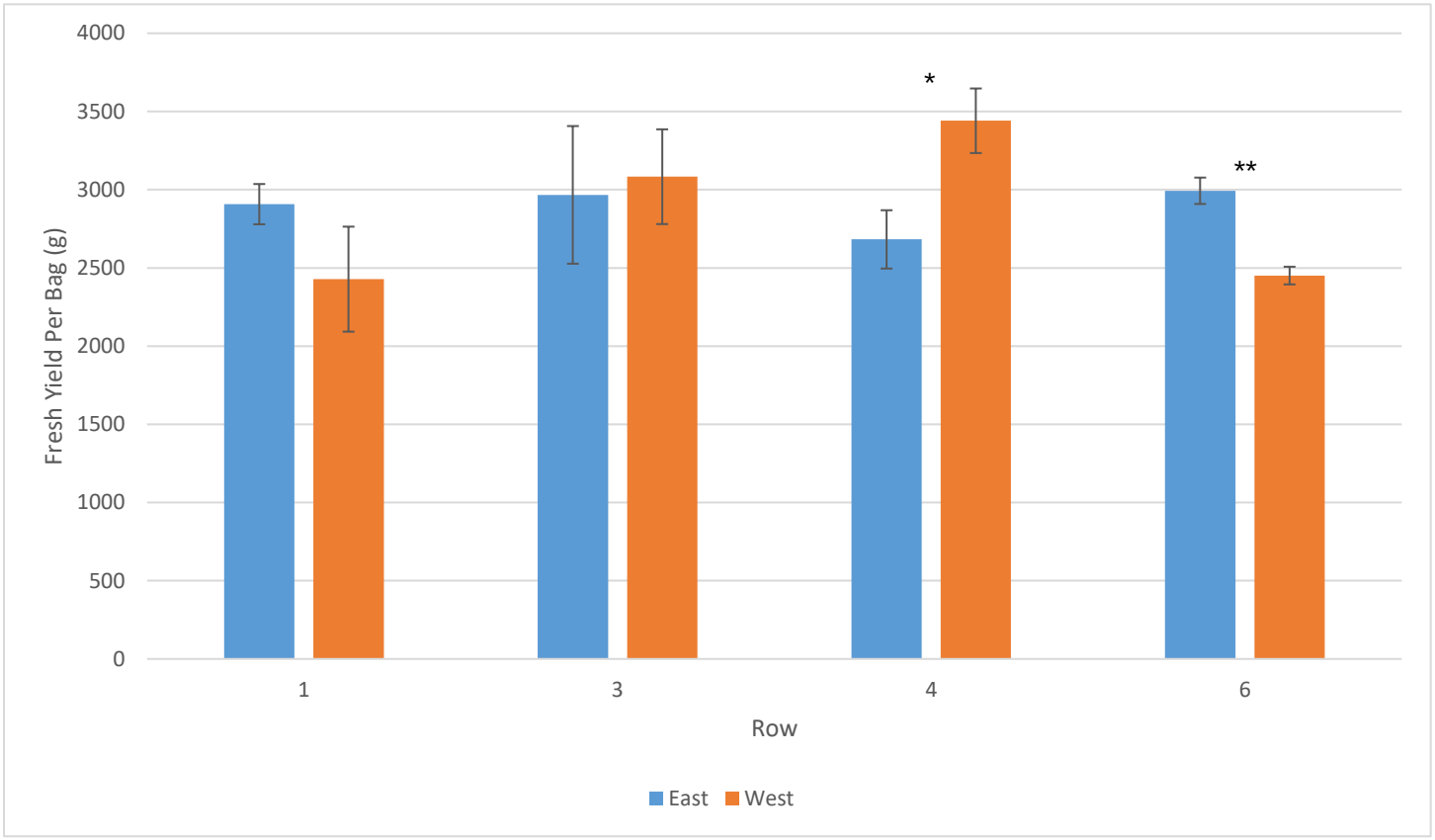

Figure 10: Strawberry yield per bag in east versus west facing plants between polytunnel rows. * = $p < 0.1$; ** = $p < 0.05$. n = 4.

Yield of individual plants was also recorded. Analysis of these plants by grouping into yield per bag identified significant differences between east and west facing plants in rows 4 and 6. Greater yields by fresh mass were identified for plants grown on the more sheltered aspect of these rows.

Discussion

Transformation experiments

Plasmid construction via Golden Gate was successful while construction via the LR clonase reaction was not

Despite several repeated efforts and the use of multiple replacement kits from the supplier, the pENTR/D-TOPO kit was found to be unsuccessful for cloning of the cDNA fragments obtained from *A. thaliana* mRNA. This was attributed to a batch of the kits being faulty as the

supplier had received several complaints of this nature from multiple labs. While the graphics in **Figure 1** looked promising initially, sequencing revealed these to be false positives. This highlights the importance of using multiple methods of verification when developing plasmids to ensure construct generation has been completely successful. Golden Gate cloning was found to be a rapid and highly successful alternative.

Moving forward, new constructs will be generated containing genes coding for green fluorescent protein (GFP) and β-glucuronidase (GUS) under the control of promoters derived from strawberry Calvin-Benson Cycle enzymes. This is to enable development of a toolbox of tissue specific promoters for use in strawberry, similar to work performed by Alotaibi et al., (2018) in wheat. Gateway Binary Vectors (pGWBs) with these genes are readily available for this cloning work (Nakagawa et al., 2007; Nakamura et al., 2010)

Regeneration and transformation of strawberry leaflets is achievable in Calypso and EMR 773-5 cultivars

12 weeks of growth on SRM was sufficient for regeneration of new leaves in both cultivars. After transplanting onto SPM, further growth and differentiation of stem and root tissue was observed. This demonstrates that these cultivars are suitable choices for transformation experiments, although further study with regenerating more commercially relevant lines would be beneficial for greater understanding of real world applications of this research. Tissue culture methods are not predicted to affect photosynthetic parameters as current literature has not identified differences between plants grown *in vitro* and *ex vitro* (Borkowska, 2001) although differences in leaf photosynthetic activity may be present during weening (Grout and Millam, 1985). Regardless, it would be prudent to consider tissue culture effects on strawberry photosynthesis to capture any changes that may be effected.

The strawberry transformation protocol has been fine-tuned and is now producing transformed plantlets for the cultivar Calypso

Use of light exposure to aid callus induction indicates the important role light plays in strawberry organogenesis (reviewed here: Schaart, 2011). Further exploration with this may aid in further improving the efficiency of this protocol for regeneration of plantlets, especially in the EMR-773 variety. Reduced light intensity may be a possible avenue for improved regeneration as suggested by Schaart, 2011. Transformation has been shown to be successful for both the EHA105 and AGL1 strains of Agrobacterium, with a range of efficiencies of 40.3 % to 73.8 %. Despite the relatively lower efficiency of EHA105 compared to AGL1, this strain still exhibits high efficiency for a plant transformation protocol and is therefore still of use in experiments involving strawberry transformation.

Transgenic lines show elevated photosynthetic traits which are correlated with increased yield

It has previously been shown that increasing capacity for NPQ leads to increases in photosynthesis and yield (De Souza *et al.*, 2022; Kromdijk *et al.*, 2016) 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 see an increase in fruit yield.

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 CBB Cycle.

Flavour experiments

Strawberry flavour compounds are highly sensitive to cultivar type, shelf life and treatment conditions

Preliminary strawberry flavour data has revealed that these experiments must be tightly controlled. It is unsurprising that different cultivars have different flavour profiles, though the greater aroma repertoire of Eve's Delight when compared to Sweet Eve remains striking (see **Figure 7**). Increased glucose content in older strawberries suggested degradation of the disaccharide sucrose. Loss of sucrose over time has been observed previously in strawberry fruit (Schwieterman et al., 2014; Watson, 2002), however this has also been accompanied by a reduction in monosaccharide hexose sugars. It is possible that this trend may emerge more

clearly beyond shelf-life (not tested in this experiment) or would be visible with a greater number of samples – the margin of error in the experiment was large (see **Figure 5**), possibly due to pick-to-pick variation of individual fruit sugars. Future work on fruit sugars should consider these points. Two different treatments of strawberry were tested when examining aroma profile: no treatment (i.e. fresh) and freeze-drying. Freeze-drying reduced the bouquet of volatiles that could be detected in Eve's Delight and did not add any new volatiles. This suggests that freeze-drying results in degradation of the extensive compliment of esters present in the fresh samples. This effect has been reported in the literature for deep frozen strawberries (Douillard and Guichard, 1990), so it is possible that freeze-drying has a similar impact. Interestingly, while some volatiles are lost during freeze-drying, Sweet Eve freeze-dried samples gained some detectable volatiles. Included in these were hexanoic acid and octanal, a carboxylic acid and an aldehyde respectively. These may be breakdown products of esters, further supporting the idea that freeze-drying breaks down esters in the strawberry fruit, although a lack of ester hydrolysis products in the freeze-dried samples of Eve's Delight does provide evidence against this theory. Taken together, it is clear that flavour compounds are highly sensitive and experiments in this field must be tightly controlled.

Field Work

Strawberry photosynthesis is enhanced in row 4, correlating with increased fresh fruit yield

It is widely accepted that photosynthetic efficiency is a limiting factor on yield potential of crops (Long et al., 2006). It follows that increased strawberry photosynthesis, as observed in polytunnel row 4, would lead to a concomitant increase in fresh fruit yield. This was derived from an increase in fruit number. Increased photosynthetic efficiency has previously been shown to accelerate developmental rate and seed number (Simkin et al., 2017b). It could therefore be postulated that the improved photosynthesis of row 4 led to an enhancement in floral initiation or allowed for assimilated carbon to be allocated to a greater number of developing fruit. To investigate this further, future work could examine the relationship between flower number, fruit fresh yield and photosynthetic efficiency to unpick these developmental patterns.

Yield is increased on more sheltered sides of rows 4 and 6

The causes of this unusual pattern of fruit growth are as yet unclear. It is possible that elevated temperature in the more sheltered phytoclimate due to absorption and release of heat from neighbouring rows created more favourable conditions for greater fruit yield. It is also notable that this effect was only observed on the eastern side of the polytunnel, perhaps indicating that this phenomenon is related to diurnal patterns of photosynthesis, such as was

observed in previous work. Future work should look at repeating this experiment during a different year to establish if these results are robust across growing seasons.

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. 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 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 observed in the field work 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.

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Appendices

Appendix 1 – Media Recipes

Appendix 1.1 – 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

Appendix 1.2 – 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

Appendix 1.3 – 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

Appendix 2 – Master Mix Recipe for Colony PCR

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

[1]Primers at 10 µg mL^{-1}. [2]Minimum primer volume of 1 µL.

Appendix 3 – Volatile Compounds from Strawberry Fruit

Compound	Present where?
Methyl butanoate	All
Ethyl butanoate	All
2-hexenal	All
Methyl hexanoate	All
Hexenyl acetate	All
Linalool	All
Hexenyl butanoate	All
Hexenol	Fresh and freeze-dried Sweet Eve, fresh Eve's Delight
Ethyl hexanoate	Fresh and freeze-dried Sweet Eve, fresh Eve's Delight
Decalactone	Fresh and freeze-dried Sweet Eve, fresh Eve's Delight
Butyl butanoate	Fresh and freeze dried Eve's Delight, freeze-dried Sweet Eve
Ethyl acetate	Fresh Sweet Eve, Fresh Eve's Delight
Nerolidol	Fresh Sweet Eve, Fresh Eve's Delight
Methyl acetate	Fresh and freeze-dried Eve's Delight
Butyl acetate	Fresh and freeze-dried Eve's Delight
Mesifurane	Fresh and freeze-dried Eve's Delight
2-heptanone	Freeze-dried Sweet Eve
Hexanoic acid	Freeze-dried Sweet Eve
Octanal	Freeze-dried Sweet Eve
Isopentyl acetate	Fresh Eve's Delight

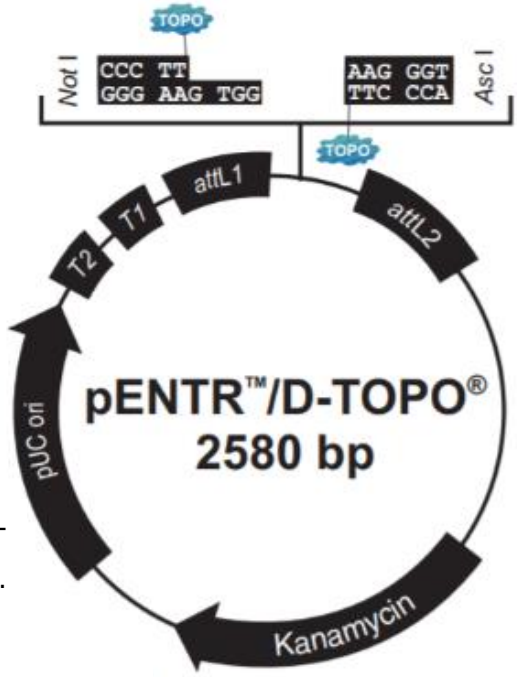
Hexyl acetate	Fresh Eve's Delight
Octyl acetate	Fresh Eve's Delight
2-methylbutyl octanoate	Fresh Eve's Delight

Supplementary Figure 1 – pENTR/D-TOPO Vector Map

Comments for pENTR™/D-TOPO®
2580 nucleotides

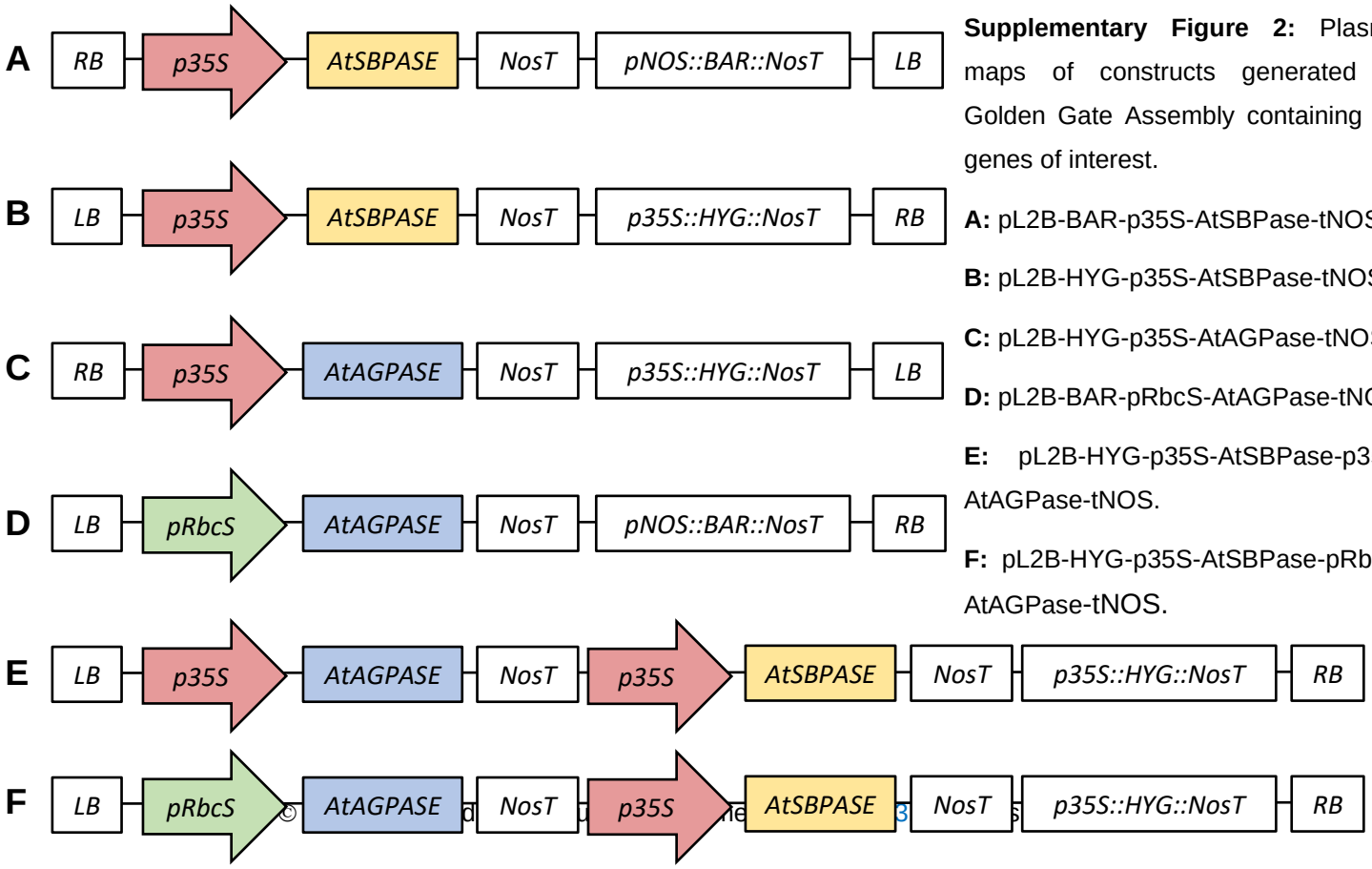
*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (c)
TOPO® recognition site 1: bases 680-684
Overhang: bases 685-688
TOPO® recognition site 2: bases 689-693
*att*L2: 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



Supplementary Figure 1: Map of pENTR/D-TOPO vector with comments on component parts. Adapted from Invitrogen manual.

Supplementary Figure 2 – Golden Gate Plasmid Maps



Supplementary Figure 2: Plasmid maps of constructs generated by Golden Gate Assembly containing the genes of interest.

A: pL2B-BAR-p35S-AtSBPase-tNOS.

B: pL2B-HYG-p35S-AtSBPase-tNOS.

C: pL2B-HYG-p35S-AtAGPase-tNOS.

D: pL2B-BAR-pRbcS-AtAGPase-tNOS.

E: pL2B-HYG-p35S-AtSBPase-p35S-AtAGPase-tNOS.

F: pL2B-HYG-p35S-AtSBPase-pRbcS-AtAGPase-tNOS.