The Significance of Non-Foliar Photosynthesis on Development and Nutritional Value in Tomato Fruit

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Thesis submitted to the University of Nottingham School of Biosciences for the degree of Doctor of Philosophy (September 2025)

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Acknowledgements

I would like to express my gratitude to the University of Nottingham BBSRC DTP and UKRI for funding this project; without their investment, it would not have been possible.

Additionally, I sincerely thank my co-supervisors, Prof. Erik Murchie and Dr Andrew Simkin. Their invaluable expertise, support, and encouragement throughout this entire journey have been pivotal in shaping this thesis. I am grateful for their constructive criticism and for challenging my thought process while providing me with the space and confidence to grow as an academic. They have been my role models as well as being the blueprint for the type of senior scientist I wish to become.

I extend heartfelt gratitude to the Murchie group, Simkin group and the Crop Science lab technicians Kamal Swarup and Kade Marshall. They have been warm, welcoming, and most of all supportive of me and my project. They were a significant factor in making my journey both enjoyable, memorable and productive during difficult days.

Last but certainly not least, I am deeply thankful to my family for their unwavering love and support. Without my mum, Sharon, and my late grandfather, Papa, nurturing my scientific curiosity and fascination with plants, none of this would have been possible. They both instilled in me that it is good to ponder, “try to see around corners”, but even better to seek answers, “learn your book”. I am truly thankful for the endless support shown by my sister, Leanne, and my brother, Dominic. They have been my source of strength and have always pushed me to go further and be confident in my thought process. They have shown me that no goal is out of reach. Furthermore, I would like to thank my girlfriend, Aileen. Without her lending an ear to my ramblings and offering valuable advice, my journey would have been much more difficult. My last thanks goes to my dog, Tank. His untiring support was invaluable; he reminded me that at no point am I alone by keeping me company during the many late nights throughout my education.

“When your belly is full, the world will look bright.” – Fred Charles ‘Papa’

Disclosure: Grammarly was used as an AI-based writing assistant solely for spelling, grammar, and style corrections. ChatGPT (OpenAI) was used to generate Figure 6.2. No AI-based tools were used to generate text, interpret data, or draw conclusions. I take full responsibility for all scientific content presented within this thesis.

List of Abbreviations

°Bx – degrees Brix
µmol m^{-2} s^{-1} – Micromoles of photons per square meter per second
3-PGA – 3-phosphoglycerate
Abs – Absorbance
ADP – Adenosine diphosphate
AFF – All-Flesh Fruit
AP2a – Apetala2a
ATP – Adenosine triphosphate
Azy – Azygous
BA – 6-Benzylaminopurine
BSA – Bovine Serum Albumin
CAO – Chlorophyll a Oxygenase
CBB – Calvin-Benson-Bassham
CEF – Cyclic Electron Flow
Chl – Chlorophyll
cm – Centimetre
CO_2 – Carbon Dioxide
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
DAF – days after flowering
dH_2O – Distilled water
DHAP – Dihydroxyacetone phosphate
DMF – Dimethylformamide
DNA – Deoxyribonucleic acid
DPA – Days Post-Anthesis
dsRNA – Double-stranded Ribonucleic acid
E4P = erythrose 4-phosphate
ETC – Electron transport chain
FBP – Fructose-1,6-bisphosphate
FAO – Food and Agriculture Organisation
FBPAa – Fructose-1,6-bisphosphate aldolase
FBPase – Fructose 1,6-bisphosphatase
FNR – Ferredoxin-$NADP^+$ reductase
Fq′/Fm′ – Effective quantum yield of photosystem II
Fv/Fm – Maximum quantum efficiency of photosystem II in a dark-adapted state
Fv'/Fm' – Maximum quantum efficiency of photosystem II in the light
g – Gram
G3P – Glyceraldehyde-3-phosphate
G6P = glucose 6-phosphate
GAP = glyceraldehyde 3-phosphate
GFP – Green Fluorescent Protein

HSP – Heat Shock Protein
IAA – Indole-3-acetic acid
IPTG – isopropyl-β-D-thiogalactopyranoside
IQR – Interquartile range
IRGA – Infrared Gas Analysis
Kg – Kilogram
L – Litre
L1 – Level 1
LB – Lysogeny Broth
LED – Light-emitting diode
LEF – Linear Electron Flow
LHC – Light-Harvesting Complexes
LHCII – Light-Harvesting Complex II
MEP – Methylerythritol phosphate
mg – Milligram
ml – Millilitre
MS – Murashige and Skoog medium
NADP – Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH – Nicotinamide adenine dinucleotide phosphate (reduced)
NFP – Non-foliar Photosynthesis
nm – Nanometre
NOS – Nopaline Synthase
NPQ – Non-Photochemical Quenching
NPTII – Neomycin phosphotransferase II
OCS – Octopine synthase
OD – Optical Density
OEC – Oxygen-evolving complex
PAR – Photosynthetically Active Radiation
PC – Plastocyanin
PCR – Polymerase Chain Reaction
PDS – Phytoene Desaturase
PFK – Phosphofructokinase
PGA – Phosphoglyceric Acid or 3-Phosphoglycerate
PIF1a – Phytochrome Interacting Factor 1a
PQ – Plastoquinone
PRKase – Phosphoribulokinase
PSI – Photosystem I
PSII – Photosystem II
PSY1 – Phytoene Synthase 1
QA – Primary quinone electron acceptor of Photosystem II
qP – Photochemical quenching
R5P = ribose 5- phosphate
RIN – Ripening Inhibitor
RISC – Ribonucleic acid-induced silencing complex
RNA – Ribonucleic acid
RNAi – Ribonucleic acid interference
RNase – Ribonuclease
ROS – Reactive Oxygen Species
RPM – Revolutions Per Minute
RuBisCO – Ribulose-1,5-bisphosphate carboxylase/oxygenase

RuBP – Ribulose-1,5-bisphosphate
SBP = sedoheptulose 7-bisphosphate
SBPase – Sedoheptulose-1,7-bisphosphatase
SD – Standard Deviation
SOB – Super Optimal Broth
SPAD – Soil-Plant Analysis Development (chlorophyll meter)
T_0 – Transformation generation 0
T_1 – Transformation generation 1
T_2 – Transformation generation 2
TCA – Timentin & Clavulanic Acid
TFM7 – A fruit-specific promoter
USD – United States Dollar
UV – Ultraviolet
WT (Azy) – Wild-type (Azygous)
X-GAL – 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside
Xu5P = xylulose 5-phosphate
ZDS – ζ-Carotene Desaturase
Δ– Change in
ΦPSII – Effective quantum yield of photosystem II

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Abstract

The primary aim of this study was to investigate whether the targeted manipulation of CBB cycle enzymes and chlorophyll partitioning could be used to improve photosynthetic performance, biomass accumulation, and metabolic composition in tomato fruit. During the early stages of fruit development, many fruits contain high quantities of chlorophyll along with the apparatus required to successfully photosynthesise. In species such as *Solanum lycopersicum* (tomato), 29% of all photosynthetic electron transport activity occurs in non-foliar organs such as the green fruit, supporting tissues and calyces. This form of photosynthesis is called non-foliar photosynthesis (NFP). Recent studies have shown that NFP, like leaf photosynthesis, significantly contributes to the production of photoassimilates. Studies have shown that improving photosynthetic efficiency in leaves can have a significant impact on biomass and nutritional quality. Similar improvements to NFP may have a comparable effect. However, NFP remains an underexplored area, despite the numerous potential benefits. The Calvin-Benson-Bassham (CBB) cycle and light-harvesting apparatus could be targeted within fruit to improve their photosynthetic capacity, efficiency, and rate. In doing so, it will aid in determining the significance of NFP.

Through genetic modification, fruit-specific promoters were introduced that allow for selective differential expression of my genes of interest, Sedoheptulose-1,7-bisphosphatase (SBPase) and Fructose 1,6-bisphosphate aldolase (FBPa), to generate novel tomato lines. My genes of interest are enzymes that catalyse a critical step in the regeneration of ribulose-1,5-bisphosphate (RuBP), necessary for continuous CO_2 fixation. Additionally, I silenced the expression of Chlorophyll *a* Oxygenase (CAO) in fruit via RNAi. This enzyme is essential for the conversion of chlorophyll *a* to chlorophyll *b*; its silencing reduces the antenna size within the light-harvesting complex. This reduction allows for photosynthetically active radiation (PAR) to penetrate further into the fruit pericarp. This potentially allows more light-harvesting complexes to participate, enhancing light use efficiency by reducing non-photochemical quenching (NPQ). These studies aided me in assessing how fruit photosynthesis contributes to developmental processes and quality traits.

This project identified that the significance of fruit photosynthesis cannot be directly inferred from leaf studies, as tomato fruit are a complex and unique regulatory environment with distinct metabolic constraints. It was identified that silencing the enzyme led to increased sugar accumulation in fruit, while overexpressing the enzyme did not enhance compositional traits

and reduced PSII efficiency. While FBPA overexpression did not alter fruit photosynthetic efficiency, a decoupling between biomass accumulation and sugar content occurred as the fruit biomass increased at the expense of soluble sugar concentration. I identified that silencing CAO in fruit has a measurable effect on carotenoid partitioning, PSII function (under specific conditions) and causes a shift in carbon partitioning away from sugar accumulation. The suggestions proposed towards further studies are of interest to the fruit industry and consumers alike.

1 Chapter 1: Introduction to Non-Foliar Photosynthesis in Tomato Fruit

1.1 Introduction

Alongside cereals, which provide most of the calories and protein for humans, yield improvements in crops such as tomatoes, cucumbers, apples, and other soft fruits have been a cornerstone of efforts to address global food insecurity. Increasing the productivity of fruit crops can play a crucial role in ensuring a stable and sufficient food supply, particularly in the face of growing demand (Fischer, Byerlee, and Edmeades, 2014). As of 2020, an estimated 811 million people, around 9.9% of the world's population, were malnourished. Unfortunately, this statistic is projected to rise due to a combination of factors, including climate change, population growth, and the loss of arable land through unsustainable agricultural practices (FAO, 2021).

However, while substantial progress has been made in increasing crop yields over the past century, especially through advances in genetics, agronomy, and mechanisation, much less attention has been paid to the nutritional value of the food being produced. For decades, the focus of plant breeding has largely prioritised traits such as productivity, pest resistance, shelf life, and aesthetics, often without much attention being paid to nutrient density (Joseph et al., 2017; DeFries et al., 2015; Graham et al., 2007; Welch & Graham, 2004). However, it must be noted that there have been some attempts with varying success involving directly increasing the nutrient density of crop products. A notable example includes Golden Rice produced within the Golden Rice Project. Golden Rice is rice that has been genetically engineered to contain β-

carotene, a precursor to vitamin A. This was achieved by transforming rice with phytoene synthase and phytoene desaturase transgenes (Beyer *et al.*, 2002; Tang *et al.*, 2009).

Globally, we face a paradox: although there is more food than ever before in terms of calories, billions of people still lack access to essential vitamins and minerals. This phenomenon has been dubbed "hidden hunger" (Muthayya et al., 2013).

This disconnect between quantity and quality is becoming increasingly problematic as a result of the needs of a projected 10.9 billion people by the year 2100 needing to be met (Lam, 2025). This will require both growing more food and food of a higher nutritional quality. The crops will need to not only feed the consumer but also nourish them to a higher degree than ever before (Roser, 2013).

In this context, tomatoes emerge as a particularly promising crop. As one of the most widely consumed fruits globally, tomatoes are already a valuable source of nutrients, including vitamin C, potassium, folate, and health-promoting compounds like lycopene and β-carotene (Perveen et al., 2015; Adalid, Roselló and Nuez, 2010; Parmar, Rajput and Johri, 2021). These nutrients are not just important for general health; they may also play preventative roles in conditions such as cardiovascular disease, certain cancers, and male infertility (Giovannucci, 1999; Williams et al., 2020). However, the nutritional potential of tomatoes is far from optimised. Tomatoes could be bred or genetically manipulated for higher micronutrient content, increased antioxidant capacity, or more favourable bioavailability of phytonutrients. This has received comparatively little investment compared to traits linked to productivity or transportability. Increasing evidence suggests that improving the nutritional quality of horticultural crops such as tomatoes is feasible and necessary, particularly as dietary patterns shift towards greater consumption of plant-based foods (Saltzman et al., 2013; Simkin, 2019; Ducreux et al., 2005; Römer et al., 2000; Díaz de la Garza, Gregory and Hanson, 2007).

To effectively combat hidden hunger, plant molecular biologists, breeders, and agricultural researchers must adopt a more holistic approach, one that integrates yield (fruit size, weight, and/or number), resilience, and nutrition. In this regard, the tomato stands out not just for its economic importance and genomic accessibility but also for its contribution as a nutritionally rich, climate-resilient, and globally relevant crop. The use of modern molecular techniques, climate-smart farming, and nutritional profiling tools will be essential to ensure that tomatoes and other underutilised photosynthesising fruit can be used to solve the issue of feeding and nourishing our rapidly increasing population.

1.2 Photosynthesis

All plants predominantly use photosynthesis, foliar or non-foliar, as a means to produce glucose, which is essential for the completion of basic cellular and molecular processes such as growth and reproduction. All organisms which capture energy in the form of light from the sun to power cellular processes in this way are called autotrophs (Sage, 2008). Autotrophs intercept light energy with organs specialised for this task, most notably leaves, which have evolved to maximise light absorption and gas exchange (Lambers, Chapin & Pons, 2008; Sage, 2008). The intercepted solar radiation is then converted into chemical energy through photosynthesis, enabling plants to produce carbohydrates that can be used immediately or stored for later metabolic needs (Goudriaan et al., 1985).

A plant's photosynthetic capacity is influenced by both internal physiological traits and external environmental factors. Internally, chlorophyll content, leaf structure, and the number and distribution of chloroplasts play key roles. Externally, environmental variables such as light intensity and quality, water availability, temperature, and atmospheric carbon dioxide concentration all strongly affect photosynthetic efficiency (Borsuk and Brodersen, 2019; Ren, Weraduwage and Sharkey, 2019a, 2019b; Wu *et al.*, 2022; Zhang *et al.*, 2022; Xu *et al.*, 2023).

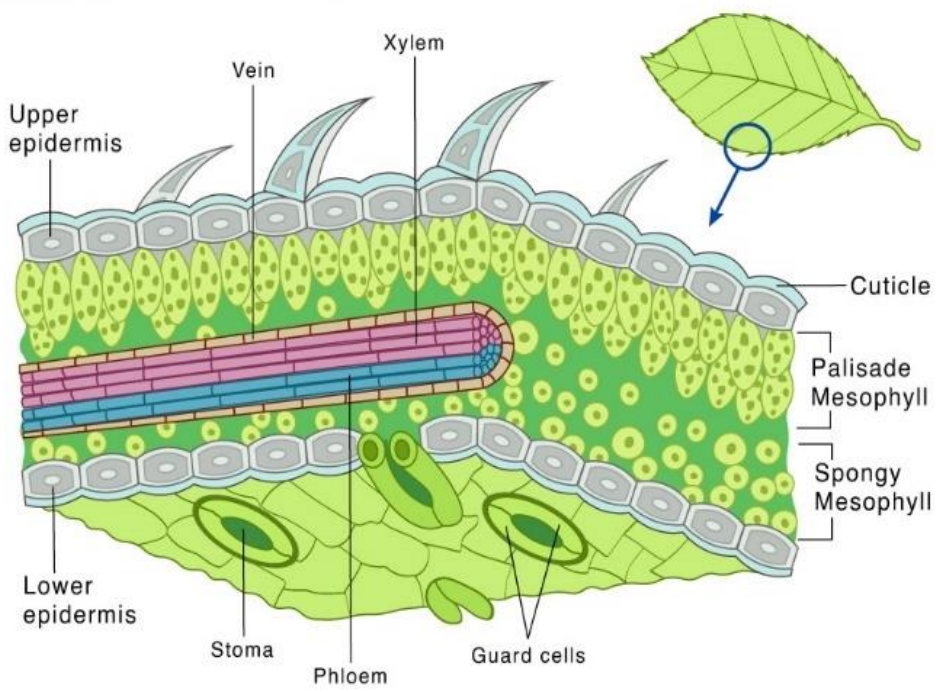

Figure 1.1. *An illustration of the anatomy of a leaf (image source: sciencefacts.net).*

Plants utilise a narrow portion of the electromagnetic spectrum, known as photosynthetically active radiation (PAR), which ranges from 400 to 700 nm. This range encompasses the blue and red wavelengths, which are most effectively absorbed and used in photosynthesis (Carruthers et al., 2001). More specifically, PAR is intercepted and utilised by specialised organelles called chloroplasts, which are typically found within cells in the palisade mesophyll (Fig. 1.1) (Williams, Gorton and Witiak, 2003). Chloroplasts contain specialised pigments called chlorophyll *a* and chlorophyll *b*, which act as the primary pigments responsible for harvesting light energy. Chlorophyll *a* is the central pigment in both photosystems and is essential for the photochemical reactions of photosynthesis, while chlorophyll *b* functions as an accessory pigment, extending the range of light absorption and transferring captured energy to chlorophyll *a*.

In addition to chlorophylls, chloroplasts contain other accessory pigments such as carotenoids, including β-carotene and xanthophylls. These pigments absorb light in regions not effectively captured by chlorophyll (mainly in the blue-green region, around 450 to 550 nm), thus enhancing the efficiency of light capture (Kume, 2017). Carotenoids also perform critical photoprotective functions by dissipating excess light energy as heat and preventing the formation of reactive oxygen species that could damage the photosynthetic apparatus (Choudhury and Behera, 2001; Latowski, Kuczyńska and Strzałka, 2011; Demmig-Adams & Adams, 1996). In some plant tissues, especially fruits and flowers, pigments such as anthocyanins may also contribute to light absorption and stress tolerance, although their role in photosynthesis is more limited and species-specific (Zheng *et al.*, 2021; Zhao *et al.*, 2022).

An essential component of photosynthesis is the stomata. Stomata are microscopic pores predominantly located on the abaxial (lower) leaf surface (Fig. 1.1). Stomata function to regulate gas exchange by allowing for the diffusion of carbon dioxide (CO_2) into the leaf, which is a necessary substrate for the CBB cycle (Harrison *et al.*, 2020; Haworth *et al.*, 2021; Lemonnier and Lawson, 2024). Simultaneously, they facilitate the loss of water vapour through transpiration, an essential process that indirectly influences nutrient uptake and leaf cooling. The opening and closing of stomata are tightly controlled by guard cells and are highly responsive to environmental stimuli such as light, humidity, CO_2 concentration, and internal circadian rhythms (Buckley, 2005; Engineer *et al.*, 2015; Sun *et al.*, 2023). Efficient stomatal regulation is crucial to balancing CO_2 uptake for photosynthesis with water conservation, particularly in arid and high-temperature environments (Sun *et al.*, 2023).

1.2.1 Chloroplast Components of Photosynthesis

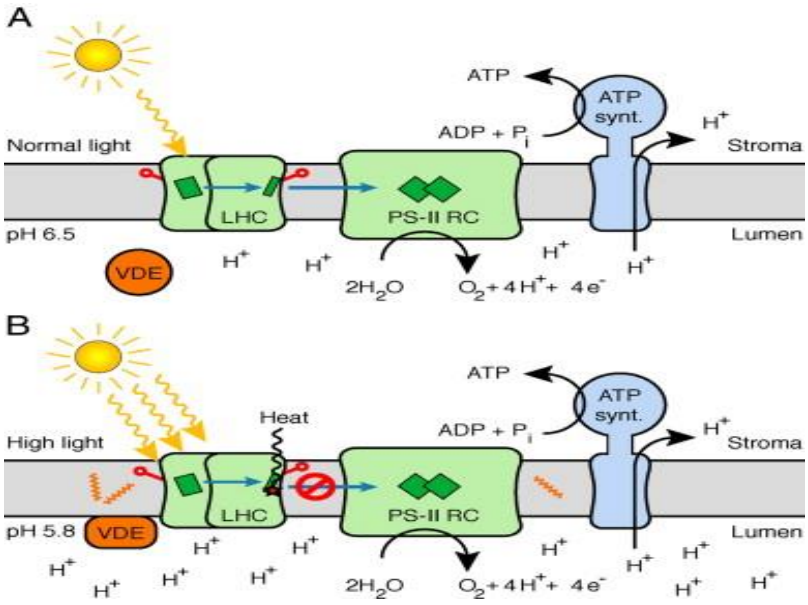

Figure 1.2. (A) *In normal light conditions, light-harvesting complexes (LHC) capture solar energy for the reaction centres of photosystems.* **(B)** *In high-light conditions, the antenna of photosystem II (PS-II) enters a quenched state and releases excess energy as heat to prevent photodamage. Under such conditions, Violaxanthin De-Epoxidase (VDE) converts violaxanthin into zeaxanthin. Image source: Barros and Kühlbrandt (2009)*

The photosynthetic process starts when light energy is absorbed by pigment-protein complexes located in the thylakoid membranes of chloroplasts. This process involves two key photosystems: Photosystem II (PSII) and Photosystem I (PSI), each surrounded by light-harvesting complexes (LHCs) that contain chlorophyll *a*, chlorophyll *b*, and carotenoids (Croce & van Amerongen, 2014). These pigments capture photons across a wide spectrum, with chlorophylls mainly absorbing red and blue light, while carotenoids broaden the absorption range into the blue-green area and also provide protection against excessive light damage (Dall'Osto et al., 2007).

The excitation energy collected by the LHCs is then transferred to the reaction centres of the photosystems through resonance energy transfer, as depicted in Figure 1.2. In PSII, this energy leads to the oxidation of water at the oxygen-evolving complex (OEC), which results in the release of protons, electrons, and molecular oxygen (Umena et al., 2011). The efficiency of this process is carefully controlled to prevent overexcitation: when excess light is present, non-photochemical quenching (NPQ) mechanisms help dissipate the extra energy as heat, thus avoiding the formation of damaging reactive oxygen species (Müller et al., 2001). Therefore,

light harvesting not only serves as the entry point for capturing solar energy but also plays a crucial role in maintaining a balance between energy absorption and the protection of the photosynthetic system.

1.2.2 Electron Transport

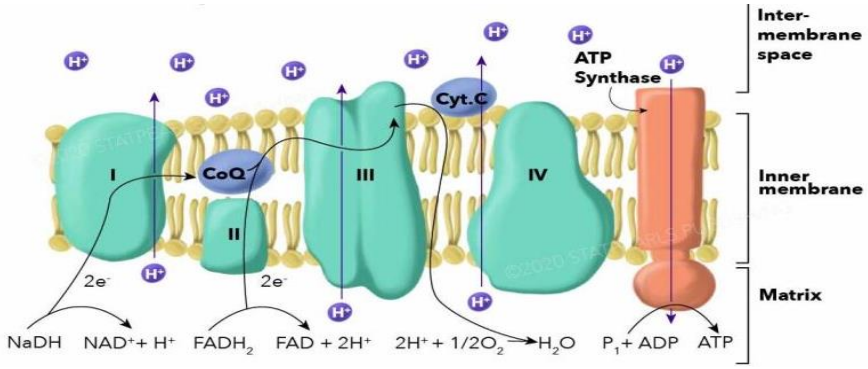

Figure 1.3. *Schematic of the mitochondrial electron transport chain (ETC) in the inner mitochondrial membrane. Electrons ($2e^-$) from NADH enter at Complex I, while electrons from $FADH_2$ enter at Complex II. Electrons are transferred via coenzyme Q (CoQ) to Complex III, cytochrome c (Cyt c), and Complex IV, where $½O_2$ is reduced with $2H^+$ to form H_2O. Proton (H^+) translocation at Complexes I, III and IV generates a proton gradient across the inner membrane that drives ATP synthesis from ADP and inorganic phosphate (P_i) via ATP synthase. Image adapted from Ahmad, Wolberg and Kahwaji (2025).*

Electrons released from water at photosystem II (PSII) enter the electron transport chain (ETC) (Fig. 1.3), which connects the two photosystems through various mobile and membrane-bound carriers. Initially, electrons from PSII are transferred to plastoquinone (PQ), then move through the cytochrome b_6f complex. This complex couples the transfer of electrons with the translocation of protons into the thylakoid lumen, as noted by Cramer et al. (2011). This process creates a proton motive force (pmf) essential for driving ATP synthesis. Subsequently, electrons are transferred to plastocyanin (PC) and then to photosystem I (PSI), where they undergo a second excitation that leads to the reduction of ferredoxin. The final step of linear electron flow (LEF) involves ferredoxin-$NADP^+$ reductase (FNR) reducing $NADP^+$ to NADPH. The ATP and NADPH produced during this process provide the necessary energy and reducing power for the Calvin-Benson-Bassham (CBB) cycle.

In addition to the ETC, plants can also perform cyclic electron flow (CEF) around PSI, where electrons return to the PQ pool instead of reducing $NADP^+$. This mechanism increases ATP production relative to NADPH, helping to adjust the energy budget of chloroplasts to meet

changing metabolic demands (Shikanai, 2014). Both ETC and CEF are finely tuned by environmental factors like light intensity, CO_2 availability, and the redox state of the stroma. Additionally, state transitions, through the reversible phosphorylation of LHCII, allow for a dynamic redistribution of excitation energy between PSII and PSI. This balance optimises electron transport and helps prevent over-reduction of the photosynthetic systems (Minagawa, 2011).

Therefore, the electron transport process acts as a crucial link between photochemistry and carbon metabolism, with regulatory mechanisms ensuring efficient energy conversion while safeguarding the integrity of the photosynthetic apparatus.

1.2.3 Calvin-Benson-Bassham (CBB) Cycle

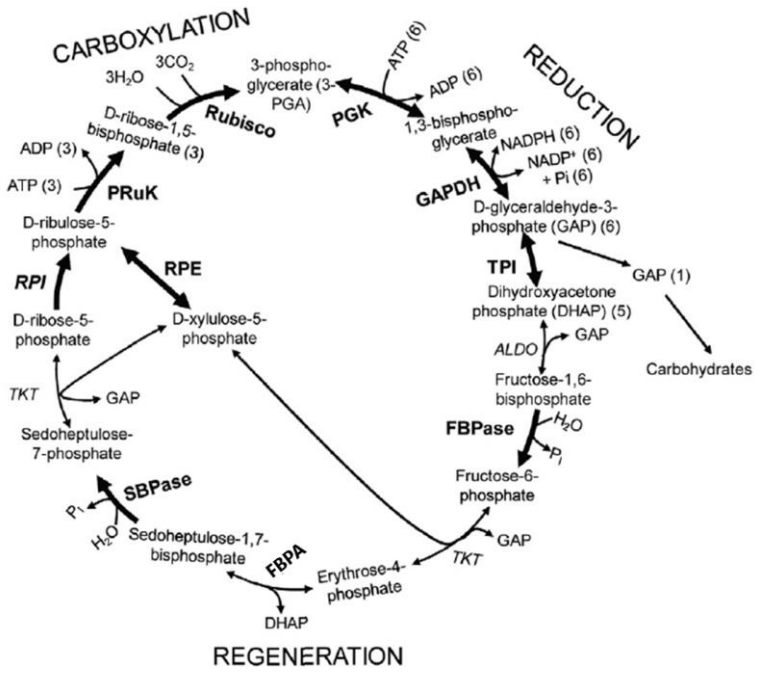

Figure 1.4. *A depiction of the CBB cycle and many of its key enzymes and products. The three stages (carboxylation, reduction, and regeneration) have been highlighted. Diagram sourced and adapted from Abebe, Wise and Skadsen (2009)*

The Calvin-Benson-Bassham (CBB) cycle involves several reactions that can be categorised into three main stages, each serving a unique purpose. These stages are the carboxylation stage, the reduction stage, and the regeneration stage, as illustrated in Figure 1.4. For the cycle to initiate, carbon dioxide from the atmosphere enters the leaves through the stomata. Once inside, CO_2 travels through intercellular air spaces, dissolves in the mesophyll apoplast, and diffuses

through both the plasma membrane and chloroplast envelope to reach the stroma, where the fixation process occurs (Flexas et al., 2008; Evans et al., 2009).

At the core of this cycle is ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which enables the carboxylation of ribulose-1,5-bisphosphate (RuBP). This reaction generates two molecules of 3-phosphoglycerate (3-PGA), marking the point at which inorganic carbon enters the cycle (Andersson, 2008). The effectiveness of photosynthesis is largely dependent on the successful incorporation of CO_2 at this stage, making Rubisco a key control point in the cycle. However, it is a relatively slow and 'error-prone' enzyme, often engaging in a competing reaction that leads to photorespiration (Bowes et al., 1971; Sharkey, 1988). Its activity is closely regulated by conditions in the stroma, including pH, Mg^{2+} levels, and the ATP/ADP ratio (Portis & Parry, 2007), as well as by Rubisco activase, which helps modify the active site to enhance catalytic efficiency (Carmo-Silva et al., 2015).

After the carboxylation process, the reduction stage utilises ATP and NADPH generated from the light reactions to convert 3-PGA into glyceraldehyde-3-phosphate (G3P). Lastly, during the regeneration stage, a series of complex rearrangements produces RuBP, enabling the cycle to continue (Raines, 2003; Heldt & Piechulla, 2011). The regulation of this cycle is highly interconnected: while Rubisco initiates the carbon fixation process, the efficiency of the subsequent reduction and regeneration phases ultimately influences the overall rate of photosynthesis. Light-dependent activation of CBB cycle enzymes through thioredoxin-mediated redox regulation and changes in stromal ions ensures a balance between the availability of energy and reductants and the requirements for carbon fixation (Michelet et al., 2013; Buchanan, 2016).

In summary, the CBB cycle operates as a precisely regulated system, with Rubisco acting as the gatekeeper for CO_2 entry into metabolic processes and the overall regulation of the cycle ensuring a steady flow and adaptability to environmental changes.

1.3 Non-Foliar Photosynthesis

Historically, the majority of photosynthetic research has focused on leaf tissues. Improving foliar photosynthesis has been widely regarded as a promising approach to enhancing crop yield and resource-use efficiency. Experimental and modelling studies have shown that increasing photosynthetic capacity through improved light harvesting, enhanced RuBisCO kinetics, or more efficient electron transport can lead to increased biomass and yield in crops (Long et al., 2006; Zhu, Long & Ort, 2010). Additionally, experiments involving genetic manipulation aimed at optimising foliar photosynthesis have demonstrated that even modest increases in carbon fixation can translate into significant improvements in productivity under both controlled and field conditions (Kromdijk et al., 2016; South et al., 2019).

A growing body of evidence suggests that other green organs, including fruits, stems, sepals, floral tissues, and some roots, can also contribute significantly to a plant's carbon economy. These non-foliar photosynthetic tissues have been largely understudied and underappreciated by the scientific community. However, their potential to enhance carbon assimilation and improve fruit development and quality is becoming increasingly recognised (Aschan and Pfanz, 2003; Liu *et al.*, 2022; Flores *et al.*, 1993).

An experiment involving keeping tomato fruit in shade showed that fruit photosynthesis contributed approximately 10–15% of the total sugar content within the developing fruit (Tanaka et al., 1974). Further studies have confirmed that tomato fruit has a functional, though lower, photosynthetic capacity when compared to leaves. For example, Piechulla et al. (1987) and Hetherington, Smillie and Davies (1998) reported that green tomato fruit possesses approximately 15-41% of the electron transport capacity observed in leaf tissues. This finding has since driven interest in whether this capacity can be improved upon through molecular or genetic approaches, potentially improving assimilate production and net sugar accumulation within the fruit.

Advances in proteomics and transcriptomics have revealed that all key components of photosynthesis, including those involved in both the light reactions and the CBB cycle (Fig. 2), are present in tomato fruits (Sagar et al., 2013; Carrari et al., 2006). Importantly, core light-harvesting complex proteins (LHCs) and photosystems I and II (PSI and PSII) have been

detected in significant quantities in developing green fruit (Sagar et al., 2013; Piechulla *et al.*, 1987; Livne and Gepstein, 1988). However, the presence of these proteins does not confirm photosynthetic functionality absolutely. To definitively establish whether fruit tissues are capable of fixing CO_2, experiments involving enzyme activity assays have been conducted. These studies have confirmed that RuBisCO is not only present but also catalytically active in tomato fruit. This greatly supports the idea that autotrophic metabolism occurs in fruit tissues during early development (Bravdo et al., 1977; Piechulla & Gruissem, 1987; Simkin et al., 2020).

As previously mentioned, photosynthesis in non-foliar tissues is not limited to fruit. Stems, particularly those that remain green throughout development, have also been shown to contribute measurably to carbon gain. In tomato, Hetherington et al. (1998) estimated that photosynthesis in the upper stem could account for up to 4% of total carbon assimilation. In some woody species, such as *Clusia minor*, covering the stems to block photosynthesis led to significant reductions in total plant biomass. Shoots and roots of stem-covered cuttings showed 23% and 34% reductions in dry weight, respectively, compared to plants with photosynthetically active stems (Kocurek et al., 2020).

Such studies allow for more attention to be placed on the enhancement of fruit photosynthesis in crops like tomatoes with the aim of significantly improving carbon assimilation and nutritional quality. The possibility of improving carbon assimilation and nutritional quality becomes especially intriguing when considering that fruit photosynthesis could be partially contributing to carbon fixation through direct CO_2 assimilation (to some capacity), but also allows for the reassimilation of carbon released through mitochondrial respiration in adjacent tissues, which is likely the primary method (Simkin *et al.*, 2020). The reassimilated carbon is refixed locally in the fruit tissue (Ávila-Lovera, Zerpa & Santiago, 2017). It has also been proposed that within fruit locular tissue, the CO_2 generated by the oxidative pentose pathway is likely reassimilated by the CBB cycle as identified in immature Brassica napus seeds (Rapeseed) (Ruuska, Schwender and Ohlrogge, 2004).

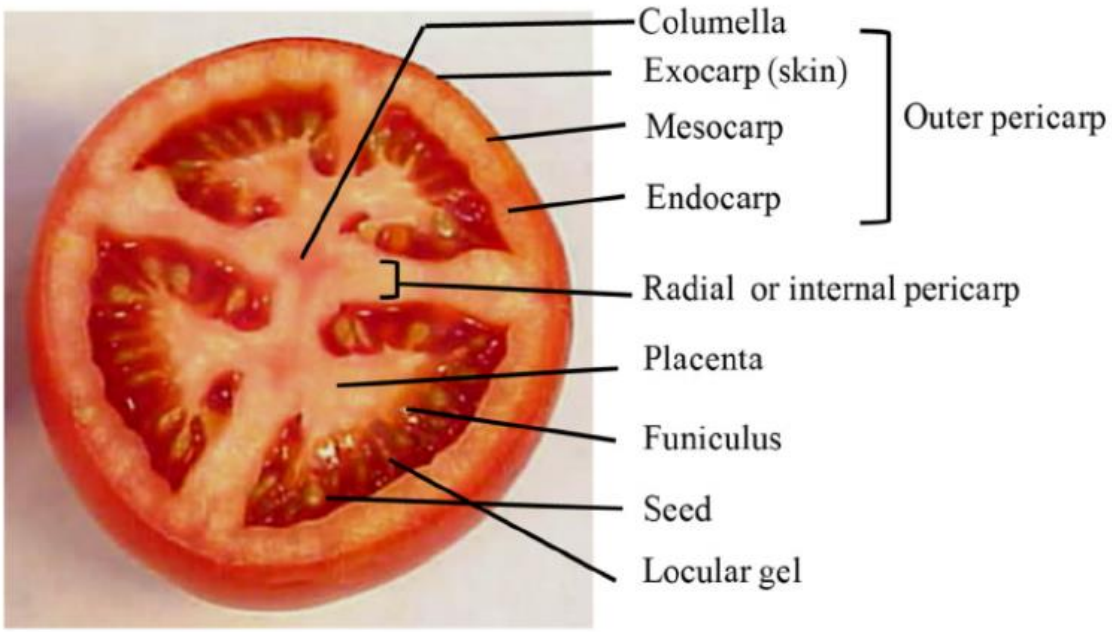

Figure 1.5. *A diagram showing the anatomy of a ripe tomato fruit (image source: Ramesh, Paul and Pandey, 2021).*

This recycling effect can increase internal carbon-use efficiency, especially under high respiratory demand during fruit development (Lemaire-Chamley et al., 2005). Despite these promising insights, the photoynthetic significance of fruits remains relatively underexplored in tomato. The mechanisms governing gas exchange in these organs are complex and poorly understood, particularly concerning stomatal function. For instance, it is often assumed that fruit photosynthesis is limited by stomatal density, as developing fruits typically exhibit only 1–10% of the stomatal number found in leaves (Sanchez et al., 2013). However, this apparent limitation may be partly misleading. The stomatal number is fixed early in development, and as the fruit expands, the increase in surface area results in stomatal dilution, creating the appearance of low stomatal density (Gibert et al., 2005). In addition to their number, the functionality and responsiveness of stomata on fruits are crucial in determining whether these structures facilitate atmospheric carbon uptake. Fich et al. (2020) noted that few studies have addressed whether fruit stomata are actively engaged in gas exchange. In some species, such as blueberry (Vaccinium corymbosum), wax deposition on the pericarp surface (Fig. 1.5) can obstruct stomatal function, effectively blocking CO_2 entry and inhibiting evaporative cooling (Yang et al., 2019). Furthermore, stomatal responsiveness to environmental cues such as light and temperature is vital, as any delay in stomatal reactions can lead to inefficient water use or inadequate cooling under heat stress (Lawson & Blatt, 2014; Fich et al., 2020). The potential multifunctionality of stomata also warrants consideration. Some have proposed that on non-

foliar organs such as fruit, stomata may not primarily serve photosynthesis but instead function in thermoregulation, providing evaporative cooling during periods of high-temperature stress (Pradeep et al., 2022; Bjerring Jensen *et al.*, 2024). This idea highlights the need for functional studies that measure stomatal conductance, aperture kinetics, and water-use efficiency in fruit tissues under varying environmental conditions. The fact that fruit lacks functional stomata additionally highlights that fruit are not organ morphologically optimised for photosynthesis and thus many of the photosynthetic hindrances are likely to be a result of this (Simkin *et al.*, 2020; Barros and Kühlbrandt, 2009).

As touched upon, there remains considerable uncertainty over the source of carbon for fruit photosynthesis. While some CO_2 may be derived from the atmosphere, other studies suggest that a significant portion originates from internal respiratory sources, especially in deeper tissues where CO_2 diffusion from the environment is limited (Henry, Furtado & Rangan, 2020). Unlike leaves (Fig. 1.1), tomato fruit (Fig. 1.5) lacks highly specialised gas-diffusion structures such as the spongy mesophyll, making vascular transport of CO_2 and internal recycling more critical for supplying RuBisCO with its substrate.

Together, these studies suggest that non-foliar photosynthesis is both real and biologically significant but largely unexploited. By further investigating and enhancing photosynthetic capacity in stems, fruits, and associated structures such as sepals, we may be able to improve crop productivity, resource-use efficiency, and even the nutritional composition of edible tissues. This reveals exciting possibilities for future work in genetic improvement, systems physiology, and the redesign of photosynthetic efficiency beyond the leaf.

1.4 Importance of Tomatoes

Solanum lycopersicum is one of the world's most economically and nutritionally significant horticultural crops. In 2022, FAOSTAT reports that the global tomato production reached approximately 186 million tonnes, highlighting its prominence in global agriculture. The crop is valued not only for its versatility in culinary applications, as it can be consumed raw, cooked, or processed into products such as sauces, pastes, and juices, but also for its substantial contribution to dietary nutrition and economic value. Processed tomato products, including

ketchup and tomato paste, account for a significant share of the global food industry, with the ketchup market alone valued at over USD 38 billion annually (*Statista Market Forecast*, 2025). In the United States, tomatoes are the second most consumed vegetable (after potatoes), which further proves their importance in the western diet (Reimers and Keast, 2016).

As mentioned, tomatoes are a rich source of lycopene, a carotenoid with potent antioxidant properties (Perveen et al., 2015; Adalid, Parmar, Rajput and Johri, 2021). Epidemiological and clinical studies have linked lycopene consumption to reduced risks of prostate and gastrointestinal cancers (Giovannucci, 1999), while recent work also suggests a potential fertility-enhancing effect in males (Williams et al., 2020). Owing to their high nutritional density and wide accessibility, tomatoes are often regarded as a cornerstone of global dietary health. Additionally, tomatoes serve as a model species in plant biology, particularly in the fields of fruit development, ripening, and genetic transformation. This organism's relatively short life cycle, sequenced and annotated genome, and responsiveness to Agrobacterium-mediated transformation have made it an ideal candidate for genetic and physiological studies (Kimura and Sinha, 2008; Shikata and Ezura, 2016).

One of the most distinctive physiological changes during tomato fruit development is the transition from chloroplasts to chromoplasts in the pericarp during ripening. In mature green fruit, chloroplasts actively photosynthesise and contribute to primary metabolism. As ripening progresses, these chloroplasts lose their thylakoid structures and transform into chromoplasts and begin accumulating high concentrations of carotenoids, particularly lycopene and β-carotene (Shikata and Ezura, 2016). This plastid transition is tightly regulated at the transcriptional level by a network of ripening regulators, including Ripening Inhibitor (RIN), Phytoene Synthase 1 (PSY1), and Apetala2a (AP2a), as well as by light-responsive transcription factors such as Phytochrome Interacting Factor 1a (PIF1a) (Llorente et al., 2016).

The improvement of our understanding of ripening and chromoplast development in tomatoes has been shown to have direct commercial implications related to disease resistance, marketability, and shelf life (Zhang et al., 2013; Klee, 2013; Galpaz et al., 2008). Beyond these traits, increasing the nutritional value of tomato fruit remains a key priority, both for consumer health and market competitiveness. Enhancing photosynthesis within fruit through the optimisation of the CBB cycle is a viable method to potentially achieve this goal (Simkin et al., 2017). Thus, manipulation of the CBB cycle represents a promising strategy to

simultaneously address yield, resilience, and nutritional enhancement in tomato fruit, benefiting industry and consumers alike.

1.5 Improving Photosynthesis in Non-foliar Organs

1.5.1 The overexpression of Sedoheptulose-1,7-bisphosphatase and Fructose 1,6-bisphosphate aldolase

As touched upon, SBPase is heavily related to photosynthetic carbon fixation as it is involved in the regeneration phase within the CBB cycle. Experimentation involving overexpressing SBPase has been carried out on tomato (Ding *et al.*, 2017), tobacco (Lefebvre *et al*., 2005), wheat (Driever *et al*., 2017), rice (Suzuki *et al*., 2019), and *Arabidopsis* (Simkin *et al.*, 2017) to explore its beneifits. Wheat research has shown that overexpressing SBPase throughout the entire plant can result in increased photosynthetic capacity by approximatly 20% and also lead to an increased total seed weight by 30–40% (Driever *et al.*, 2017). In some cases, it also increased chilling tolerance by reducing electrolyte leakage by 11% in comparison to wild-type (Ding *et al.*, 2017). These findings are significant as they show that the natural abundance of SBPase limits the CBB cycle and that with the use of genetic modification, this bottleneck can be overcome to generate crops with greater yields (Raines, 2022).

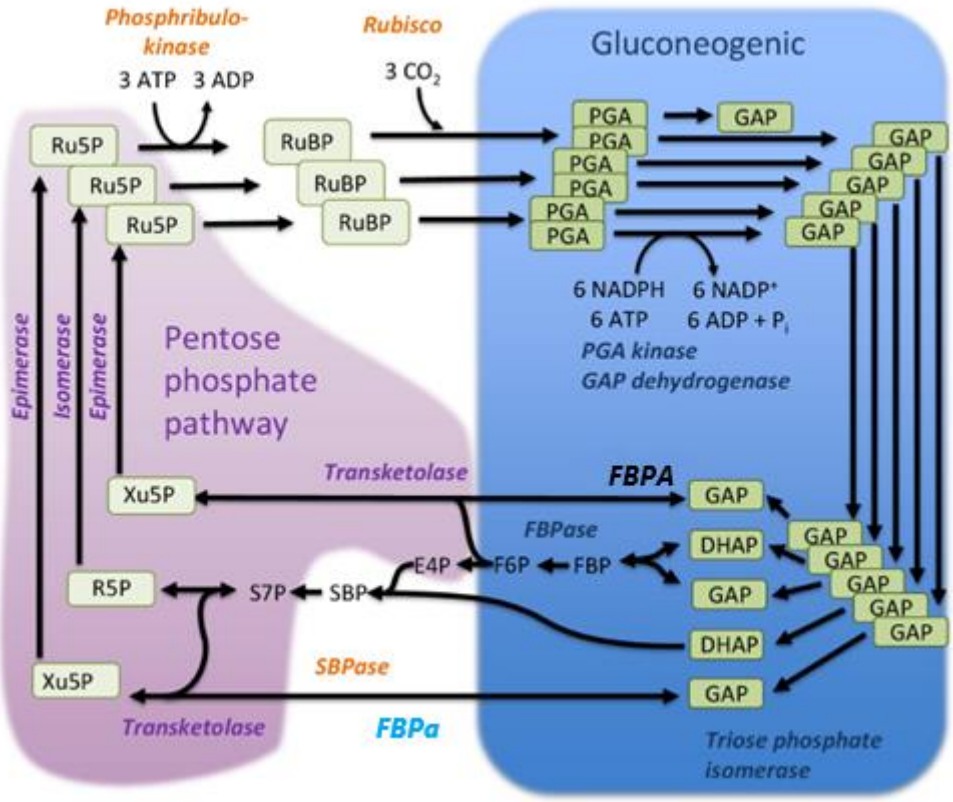

Figure 1.6 *A depiction of the Calvin-Benson cycle reactions spearated in two steps, conversion of pentose to triose (top) and triose to pentose (bottom). Reactions in the purple area are pentose phosphate pathway reactions, and in the blue area are gluconeogenic reactions. DHAP = dihydroxyacetone phosphate, E4P = erythrose 4-phosphate, FBP = Fructose-1,6-bisphosphate , G6P = glucose 6-phosphate, GAP = glyceraldehyde 3-phosphate, R5P = ribose 5-phosphate, Ru5P = ribulose 5-phosphate, SBP = sedoheptulose 7-bisphosphate, Xu5P = xylulose 5-phosphate. Image adapted from Sharkey (2019).*

There are several specific genes related to the production of crucial enzymes within the CBB cycle that may prove to be essential in improving crop yield and nutritional value (Chattopadhyay *et al.*, 2021; Simkin *et al.*, 2020). The upregulation of enzymes such as SBPase may prove to help increase nutritional value and yield in soft fruit (Cai *et al.*, 2022; Simkin *et al.*, 2020). These enzymes were identified as being rate-limiting within the CBB cycle through studies involving enzyme kinetics and evolutionary algorithms. These studies have shown that the overexpression of SBPase is a promising target for gene expression experiments in the bid to increase the efficiency of the CBB cycle and thus improve yield (Zhu *et al.*, 2007; Raines, 2022).

As briefly touched upon, research conducted by Ding *et al.* (2016) has demonstrated that tomato plants with increased SBPase activity (55–139% of wild-type level) exhibit greater

chilling tolerance. This was heavily indicated by a higher quantum efficiency of photosystem II being achieved than expected after a period of chilling; in addition, a reduced electrolyte leakage by 11% and increased RuBP regeneration were observed.

Minor changes to the activity of SBPase can have the ability to significantly alter a plant's photosynthetic capacity (Harrison *et al*., 1997; Ding *et al.*, 2016). Mechanistically speaking, the overexpression of SBPase is likely to enhance the dephosphorylation of sedoheptulose-1,7-bisphosphate (SBP) to sedoheptulose-7-phosphate (S7P), thus relieving a key regulatory bottleneck in the regenerative phase (Figure 1.6) (Raines, 2003; Lefebvre *et al.*, 2005). This will promote an increase in flux through transketolase-mediated reactions and accelerate the conversion of S7P and glyceraldehyde-3-phosphate (GAP) into downstream intermediates (e.g. erythrose-4-phosphate, fructose-6-phosphate) (Raines, 2011; Woodrow and Berry, 2003). In turn, this may boost ribulose-5-phosphate (Ru5P) regeneration and ribulose-1,5-bisphosphate (RuBP) availability (Lefebvre et al., 2005; Raines, 2003).

According to Ding *et al.* (2016), a reduction in SBPase activity has more of an effect on photosynthetic ability than that of Rubisco. A 50% reduction in the level of Rubisco can have an insignificant effect on a plant in moderate light and temperature conditions. However, under high-light conditions, Rubisco's reduced activity becomes proportionately evident (Quick *et al*., 2005). Additionally, large reductions in the levels of glyceraldehyde-3-P dehydrogenase, and phosphoribulokinase (PRKase) do not significantly limit photosynthetic capacity. This strongly suggests that these enzymes exist in excess and that a plant's photosynthetic capacity is not highly sensitive to the level of such enzymes (Quick *et al*., 2005).

As stated, the overexpression of SBPase not only results in increased photosynthetic ability but also increased abiotic stress resistance. For example, overexpression of this enzyme in rice has been shown to increase resistance to salt stress. Transgenic lines showed a near 100% recovery of the rate of CO_2 assimilation following salt concentration treatments, whereas WT lines showed ~30% (Feng *et al.*, 2007). Additionally, Feng *et al.* (2007) showed that transgenic rice overexpressing SPBase is more resistant to high temperatures. This was shown by the transgenic lines completely recovering after 2 hours of exposure to 45°C, whereas wild-type plants showed only approximately 20% recovery. This seems to be facilitated by the upregulated SBPase allowing for increased regeneration of RuBP and thus an increase in metabolic flux (Figure 1.6) (Feng *et al*., 2007; Feng *et al*., 2007)

Fructose-1,6-bisphosphate aldolase (FBPa) is a glycolytic enzyme which functions to catalyse the reversible aldol cleavage of fructose-1,6-bisphosphate (FBP) into two triose phosphates: glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (Figure 1.6) (Lv *et al.*, 2017; Simkin *et al.*, 2017). In tomato (*Solanum lycopersicum*) and other plants, FBPA is found in both the chloroplast and cytosol. Cytosolic FBPA is expressed in leaves and fruit tissues, particularly in green immature fruit, where it supports metabolic flexibility during high demand for sugar, energy, carbon partitioning (storage/transport) and biomass accumulation (Lv *et al.*, 2017; Schnarrenberger and Krüger, 1986). Similarly to that of SBPase, when FBPA is overexpressed within the leaves, it has also been shown to increase chilling tolerance (Cai *et al.*, 2022). The overexpression of FBPa is likely to enhance the production of dihydroxyacetone phosphate (DHAP) and GAP to form fructose-1,6-bisphosphate (FBP) (Figure 1.6). This may have the effect of increasing carbon throughput from triose phosphates into the regenerative pathway. This could drive an increased substrate supply for SBP production, thus indirectly improving SBPase activity by reinforcing flux through the SBP to S7P step (Uematsu et al., 2012; Simkin et al., 2017; Lefebvre *et al.*, 2005).

Moreover, the silencing of FBPA has also been shown to cause a reduction in chill tolerance in tomato plants (Cai *et al.*, 2018). Thus, the overexpression of FBPA in fruit may be a means to reduce yield losses in an industrial setting if the developing fruit is subjected to suboptimal temperatures for brief periods.

In the fruit tissue, FBPA is involved in photosynthesis, and local sugar metabolism contributes to early fruit development (Cai *et al.*, 2022). Overexpressing FBPA in fruit is a promising strategy to increase metabolic flux through triose phosphate-dependent pathways. Increasing the metabolic flux within fruit tissue could be a means to enhance sugar accumulation, promote fruit growth, and improve nutritional value (Uematsu *et al.*, 2012; Cai *et al.*, 2022).

In developing tomato fruit, chloroplasts maintain photosynthetic activity during early growth stages and contribute locally fixed carbon to primary metabolism (Hetherington, Smillie and Davies, 1998; Lytovchenko *et al.*, 2011). However, the overall capacity of the CBB cycle in fruit tissue is limited compared to leaves, as the leaves are highly optimised for photosynthesis, as it is their primary function (Simkin *et al.*, 2020). Metabolic bottlenecks can arise in fruit due to restricted enzymatic capacity and photosynthetic feedback inhibition (Simkin *et al.*, 2015). Overexpression of FBPA is expected to reduce FBP accumulation and facilitate continuous triose phosphate turnover, preventing feedback inhibition of upstream enzymes such as

phosphofructokinase (PFK) or fructose-1,6-bisphosphatase (FBPase), and sustaining RuBP regeneration (Uematsu *et al.*, 2012; Lee *et al.*, 2020). The increase in enzymatic throughput could enable more efficient fruit photosynthesis under suboptimal light and gas exchange conditions.

FBPA overexpression may also enhance sucrose biosynthesis. Increased soluble sugar levels not only contribute directly to fruit sweetness and consumer appeal but also serve as metabolic precursors for amino acids, organic acids, and antioxidants such as ascorbate. Additionally, sugar accumulation modulates fruit osmotic potential, which in turn drives cell expansion and promotes water and solute import (Chen *et al.*, 2021; Beauvoit *et al.*, 2014). As a result, elevated FBPA activity may indirectly stimulate both fruit growth and nutritional quality. The enhanced metabolic flux through central carbon pathways could also feed into the biosynthesis of secondary metabolites such as lycopene and flavonoids, compounds of nutritional and commercial importance (Bovy, Schijlen and Hall, 2007; Alves *et al.*, 2020).

The effectiveness of FBPA overexpression in fruit may depend on the coordinated regulation of several enzymes across numerous related metabolic pathways (Raines, 2011). . The overproduction of triose phosphates without a corresponding increase in their downstream use could lead to metabolite imbalances or an increase in redox stress. Therefore, success may rely on fine-tuned spatial and temporal control, potentially using fruit-specific or developmentally regulated promoters to avoid distress in non-target tissues. The co-overexpression of FBPA with proteins involved in sucrose synthesis and electron transport, such as cytochrome C6 and a cyanobacterial membrane protein named ictB, has been shown to improve CO2 assimilation. These may help to further optimise the metabolic benefits (Raines, 2022; Nookaraju *et al.*, 2010; Croce et al., 2024).

In summary, targeted overexpression of either SBPase or FBPA in tomato fruit is a rational metabolic engineering approach that is likely to enhance endogenous photosynthetic productivity, boost sugar content, and support fruit growth. By increasing RuBP regeneration and increasing metabolic flux within the fruit, this strategy addresses both developmental and compositional traits, with potential applications for improving crop quality and yield.

1.5.2 Alterations to the Light-Harvesting Machinery

Chlorophyll *a* oxygenase (CAO) is an enzyme is involved in the conversion of chlorophyll *a* to Chlorophyll *b* (Tanaka *et al.*, 1998). These chlorophyll molecules are essential pigments required for photosynthesis within green algae, terrestrial plants, and photosynthetic prokaryotes (Tanaka *et al*., 1998).

Chlorophyll *a* can be said to be the primary pigment of photosynthesis. It can be found in the antenna complexes within photosystems P680 and P700 (Panawala, 2017). Chlorophyll *b* can be said to be an accessory pigment with the function of allowing for the capture and utilisation of a wider spectrum of light. The energy captured by Chlorophyll *b* is then passed on to Chlorophyll *a* (Panawala, 2017). As touched upon, their function is to absorb light at specific wavelengths within the antenna complexes. The captured energy is then transferred along the electron transport chain to power various reactions and processes (Tanaka *et al.*, 1998).

Studies have shown that the ratio of *a* to *b* chlorophyll molecules can differ when the plant is experiencing different light conditions (Tanaka *et al.*, 1998; Sakuraba *et al.*, 2010). For example, in high light, the *a* to *b* ratio is higher than when the plant is experiencing low-light conditions. Studying the effect of chlorophyll *a* to *b* ratios is important as it can aid our understanding of how plants can adapt and evolve to survive in different conditions (Tanaka *et al.*, 1998).

Experimentation involving the overexpression of CAO in *Arabidopsis thaliana* has been shown to increase the size of the antenna complex within photosystem II. More specifically, the overexpression of CAO increased the antenna complex by 10-20%. This highly suggests that chlorophyll *b* biosynthesis is linked to antenna size within the light-harvesting complex (Tanaka *et al.*, 2001).

Decreasing antenna size within *Solanum lycopersicum* via gene silencing is likely to have some advantages, as most tomato cultivation occurs within greenhouses where light conditions are optimal. Decreasing the antenna size is expected to increase the photosynthetic rate as antennae with smaller optical cross-sections allow for a higher degree of utilised light, as reduced absorptivity per antenna allows for greater light penetration through the canopy, thus increasing light utilisation efficiency throughout the plant as a whole (Friedland *et al.* 2019; Engelmann *et al.*, 2005).

It has been demonstrated by Song et al. (2017) and Friedland et al. (2019) that overexpressing CAO to increase antenna size can have adverse effects on photosynthesis in high-light conditions. This is due to the chloroplasts having an enhanced ability to intercept a greater

quantity of photons, thus triggering a plant's mechanisms used to avoid light damage at lower light intensities than wild-type. In essence, it can be said that plants engineered to have larger antenna complexes have a lower light saturation point. This saturation point is reached when more energy is captured than the downstream electron transport chain can use to power reactions at any one point (Friedland *et al.,* 2019).

However, there is a critical threshold below which antenna downsizing becomes detrimental. If the optical cross-section is too small, light capture becomes limiting, reducing photosynthetic rates and ultimately decreasing biomass and yield. Therefore, an optimal antenna size must balance sufficient photon interception with efficient distribution of light throughout the canopy. Fine-tuning the size of the light-harvesting apparatus to match the expected light environment is essential for maximising photosynthetic efficiency and crop productivity (Friedland et al., 2019).

1.6 Technical Challenges

It has proven difficult to determine the carbon assimilation pathway in non-leaf tissues, which is a result of its complexity (Simkin *et al.*, 2020). Typically, the method used to determine the carbon assimilation pathway in leaf tissue involves the use of a carbon isotope called carbon-13. This isotope is relatively uncommon in the atmosphere and is both non-disruptive and traceable within plants; thus, it is often used to help identify how and where carbon is used in an organism (Hama *et al.*, 1983). The method of using ^{13}C has proven to be difficult in determining the carbon assimilation pathway in non-leaf tissues, as there are many biochemical steps present in these tissues that have the potential to discriminate against carbon isotopes, specifically the photosynthesis dark reactions and carbon recapture from respiration (Henry, Furtado & Rangan, 2020).

Additionally, the difficulty of studying NFP is increased due to no direct methods being available that can help detect and determine the quantity of carbon released via respiration by leaves and then recaptured by photosynthesising non-leaf tissues (Song *et al.,* 2017).

Henry, Furtado & Rangan (2020) mention that gas exchange studies carried out in non-leaf tissue only measure the net capture and/or production, not CO_2 recapture rates.

As a result, new methods will have to be generated to further our understanding of the complex pathways involved. Gas exchange measurements in non-leaf tissues cannot provide a direct and full report on the rate of carbon capture by leaves and then recapture by cells within the fruit.

Leaf CO_2 measurements are conducted with the use of infrared gas analysis (IRGA), which involves the leaf material being enclosed within an airtight container (Simkin *et al*., 2020). This is done so that differences in the composition of the gas within the chamber can be measured. Simkin *et al*. (2020) highlight that this method is problematic when used to measure fruit photosynthesis, as commercially available chambers are typically too small to be used to contain fruit. Larger chambers can be designed and built to accommodate fruit; however, this comes with its problems. Larger chambers have greater volumes, which makes it difficult to measure changes in gas differentials as a result of slow gas mixing. In addition, problems arise with temperature control regarding large-volume chambers.

Lastly, Simkin *et al*. (2020) propose that the use of 3D scanners to aid the calculation of fruit area may greatly increase the accuracy of results. The use of traditional measurement methods, such as using a ruler, can cause a miscalculation that can significantly skew results. For example, an underestimation of area can result in an overestimation of gas exchange, thus decreasing the accuracy and validity of results.

1.7 Thesis Aims

Many would agree that it would be erroneous to disregard NFP as insignificant, even when compared to foliar photosynthesis. To date, research has suggested that bettering our understanding of the significance of NFP is likely to be financially beneficial to growers and both financially and nutritionally beneficial to the public. However, in order for these groups to reap these benefits, more scientific attention must be paid to this topic (Simkin *et al*., 2020). There are likely many ways in which NFP can potentially be improved upon. For example, specific genes which are within rate-limiting reactions within the CBB cycle, such as SBPase and FBPa, can be upregulated to increase a plant's carbon capture ability (Simkin *et al*., 2020). Moreover, further studies could be conducted to determine which other genes within non-foliar organs should be differentially expressed to benefit photosynthetic ability. For example, the gene governing sepal size could be differentially expressed to make its contribution to carbon

capture more apparent. This could pave the way for future studies to fine-tune the sepals to maximise the yield and nutritional value of the fruit. Additionally, studies focusing on the introduction of foreign genes through genetic modification to improve NFP may generate interesting results.

We have yet to fully explore the effects of improving the light-harvesting ability of fruit (Ernesto Bianchetti *et al.*, 2018; Simkin *et al.*, 2020). There is potential to fine-tune this aspect of fruit to optimise them to the light environment they will be predominantly experiencing during growth (Friedland *et al.*, 2019). However, this potential cannot be fully realised without further experimentation focused on determining its feasibility and whether there are any significant trade-offs. There may be numerous benefits that could benefit agriculture and consumers by decreasing the time of development and increasing nutritional quality Simkin *et al.*, 2020).

Many questions must be answered before the creation and commercialisation of highly photosynthetically efficient tomato plants. Answers must be found involving NFP's significance, possible trade-offs, effect on the market/society, and whether the research can be implemented on other crops. The answers to these questions are essential as they will determine the success of this research topic as a whole and as a means to alleviate the social and economic difficulties that are arising as a result of climate change and the increasing global population.

In summary, this thesis aims to determine the significance and contribution of NFP in tomato fruit while exploring the feasibility of its improvement. This will be conducted through the design and production of transgenic plant lines that differentially express genes involved in photosynthesis within the fruit. Additionally, a thorough physiological study will be conducted to investigate the impacts of these modifications.

I hypothesise that fruit-localised alterations to the abundance of photosynthesis-related enzymes, such as SBPase and FBPa, will correlate with the photosynthetic rate and capacity in a manner previously observed in leaf studies. Specifically, as observed in leaf tissue, I expect that upregulation of these enzymes will lead to an increase in photosynthetic performance (Lefebvre *et al.*, 2005; Driever *et al.*, 2017; Uematsu *et al.*, 2012; Cai *et al.*, 2022), while silencing them will result in a decrease (Obiadalla-Ali *et al.*, 2004; Lawson *et al.*, 2006; Ding *et al.*, 2016)

Furthermore, I hypothesise that reducing the antenna size in fruit via RNAi-CAO (iCAO) will significantly decrease NPQ values observed during higher irradiances and at the onset of light.

I hypothesise that this will have a compounding effect, resulting in fruit with a higher biomass as a result of increased photosynthetic efficiency.

In summary, this thesis aims to determine the significance and contribution of NFP in tomato fruit while exploring the feasibility of its improvement. This will be conducted through the design and production of transgenic plant lines that differentially express genes involved in photosynthesis within the fruit. Additionally, a thorough physiological study will be conducted to investigate the impacts of these modifications.

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2 Chapter 2: Generation of Transgenic Microtom Lines

2.1 Introduction

This chapter outlines the methods used to generate novel transgenic Tomato lines for molecular and phenological studies, aiming to determine the contribution of fruit photosynthesis and its improvement. Tomato transformation is not a scientifically novel concept, and numerous protocols exist. However, not all protocols are created equal, as some are more suitable for specific tomato varieties and growth conditions. The method used below has been adapted from numerous methods and optimised for speed, repeatability and cost. These methods were used to generate the transgenic tomatoes containing my novel constructs.

2.2 Methods

2.2.1 Synthesis and Preparation of the Construct Subparts (Level 0 Modules)

All level 1 modules required for the generation of this construct were synthesised and provided by NBS Biologicals (UK). The Level 1 destination vector assembled Kanamycin resistance Level 1 module, and the Level 1 and 2 linkers were provided by the University of Essex (UK).

2.2.2 Assembly of the Level 1 Construct

The level 1 construct was generated via the assembly of level 0 modules with the use of Golden Gate Molecular Cloning. This method of molecular cloning was established by Weber *et al*. (2011).

The construct was generated through the addition of 1 µL of Level 1 destination vector (100 ng μl^{-1}), 1 µL of each Level 0 (containing the selected promoter, coding sequence, and terminator) (100 ng μl^{-1}), 1.5 µL of 10x NEB T4 buffer, 1.5 µl of 10x BSA, and 1 µL NEB T4 DNA Ligase to a PCR tube. The reaction mixture also contained 1 µL of the level-specific restriction enzymes BsaI and distilled water (dH_2O) to bring the reaction volume to 15 µL. The digestion and ligation were performed in a thermocycler using the following program: 25 cycles of 37 °C for 3 minutes and 16 °C for 4 minutes, 1x cycle of 50 °C for 5 minutes, and 1x cycle of 80 °C for 5 mins. For a more detailed set of instructions, see 'A Modular Cloning System for Standardised Assembly of Multigene Constructs" by Weber *et al*. (2011).

2.2.3 Transformation and Screening of *Escherichia coli*

Once the assembly of the level 1 (L1) construct was completed, chemically competent *Escherichia coli. (E. coli)* cells (in-house made DH5α) were transformed with the L1 construct using a heat shock method developed by Froger and Hall (2007). Tubes containing the mixture were then placed on ice for 2 minutes before being transferred to a water bath or heating block for 45 seconds, set to 42°C. The tubes were then immediately returned to the ice for an additional 2 minutes. 300 µL of Super Optimal Broth (SOB) or 115 µL of high salt Luria–Bertani Lysogenic Broth (LB) was added to the cells before they were left to incubate for 1 hour at 37 °C. The cells were thoroughly mixed via pipetting before adding 100 µL of the broth to a high salt LB agar plate containing ampicillin (100µg mL^{-1}), X-gal (1.5µL mL^{-1}) and IPTG (1.5µL mL^{-1}) to permit blue/white selection of colonies. After incubation on a high salt LB agar plate containing ampicillin (100µg mL^{-1}), X-gal (1.5µL mL^{-1}) and IPTG (1.5µL mL^{-1}), 3 individual white colonies containing the putatively correctly assembled L1 construct were isolated and then streaked into 3 separate regions on a second plate containing 100µg mL^{-1} of ampicillin.

2.2.4 Amplification, Isolation, Quantification and Confirmation of the Level 1 plasmid

Each isolated single white colony was used to inoculate approximately 5 mL of high salt LB broth containing 100µg mL^{-1} of ampicillin, then was placed into a shaker set to 180 RPM and 37 °C overnight.

The plasmids were extracted using a NucleoSpin® plasmid kit (Machery-Nagel) following the manufacturer's instructions, using sterile Milli-Q® Ultrapure water instead of the elution buffer. The concentration of the extracted plasmids was measured using Nanodrop 1000 (ThermoFisher Scientific). Extracted plasmids were diluted to 100ng μL^{-1} for further processing.

Level 1 plasmids were subjected to DraIII restriction enzyme digestion to ensure correct assembly. The Level 1 vectors contained the restriction site CACNNNGTG for DraIII digestion. 17 µL of plasmid was incubated with 1 µL of DraIII and 2 µL of 10x rCutsmart buffer for 1 hour at 37 °C. Samples were run on a 1% agarose gel at 160 volts for 1 hour to 45 minutes. A 1kb ladder was used to aid the estimation of the number of base pairs in the bands produced. If the bands were situated where they were expected, this confirmed them to be likely to be assembled correctly.

2.2.5 Assembly of Level 2 Modules

The level 1 plasmids that displayed the correct banding pattern were then used for level 2 (L2) assembly. Level 2 constructs were assembled in the same manner described in section 2.2.2; however, the restriction enzyme BbsI was used in place of BsaI. Post L2 plasmid assembly, high-efficiency NEB® 10-beta Competent *E. coli* cells were transformed via the heat shock method (see section 2.2.3). Once the cells were transformed and incubated for 1 hour, they were plated onto high salt LB agar plates containing kanamycin (100µg mL^{-1}) and incubated overnight at 37 °C.

Only successfully transformed *E. coli* cells were selected for amplification, isolation, and quantification. Transformed cells were selected over untransformed cells by using the red/white phenotype selection method (Fig. 2.5). Only colonies exhibiting a white phenotype were isolated, as this suggested that the canthaxanthin biosynthesis operon was interrupted by the insertion of the cassette into the L2 destination vector. Once transformed, the colonies were isolated, amplified and quantified using the method stated within this section.

2.2.6 Sequencing of the Assembled Plasmid

The plasmids were sequenced by Eurofins Genomics using their "TubeSeq Supreme" service. Plasmids were prepared for sequencing, accompanied by the forward primer (L2-F: TGG-CACATACAAATGGACGAACGG) and reverse (L2-R: ATGGGCTGCCTGTATCGAG-TGG) primer as per the supplier's instructions before being sent. These primers allowed for the sequencing of the level 2 module. The binding sites for these primers in each construct can be seen in Figure 2.4; the sites are labelled L2-F and L2-R. Only correctly assembled Level 2 plasmids were taken forward for plant transformation.

2.2.7 Transformation of Agrobacterium tumefaciens

The *Agrobacterium tumefaciens* strain EHA105 was chosen to mediate the transformation of my tomato tissue. Transformation was facilitated through the use of a freeze-thaw method. This method involved thawing frozen (-80°C) *A. tumefaciens* suspended in 25% glycerol on ice until the bacterial suspension completely melted. Then 5µL of the plasmid of interest at a concentration of 100ng mL^{-1} was pipetted into the *A. tumefaciens.* Agrobacterium and the plasmid of interest were flash-frozen by dropping them into liquid nitrogen for approximately 5 minutes and then placed in a heating block or water bath at 37 °C for 5 minutes.

The cells were then used to inoculate 700µL of low salt LB broth, and then were placed into an incubator set to 180 RPM and 28°C for 2 hours. It was then placed into a centrifuge set to 5000G for 5 minutes to form a pellet. Approximately 600µL of the supernatant was carefully decanted. The pellet was then resuspended in the remaining 100µL of broth. This was then used to inoculate a low salt agar plate containing rifampicin (100µg mL^{-1} and kanamycin concentration of 50 µg $mL^{-1,}$ which was then placed into an incubator set to 28°C for 48-72 hours. This protocol was named "protocol 2.5".

The transformed *A. tumefaciens* was glycerol-stocked for later use. This was conducted by first inoculating 5mL of low salt LB broth containing rifampicin (100µg mL^{-1}) and kanamycin (50µg mL^{-1}) with 3 colonies. The inoculated broth was incubated at 28°C for 24 hours before being spun down at 3000 RPM for 4 minutes. Once pelleted, the supernatant was discarded, and the pellet was suspended in 1.5 mL of sterile 50% glycerol. The *A. tumefaciens* glycerol mix was pipetted into a cryotube and placed into a -80°C freezer.

2.2.8 Generation and Preparation of Tissue for Transformation

The method of seed sterilisation used involved placing the desired number of seeds into a Falcon tube, then adding enough 15% v/v household bleach (or 5% sodium hypochlorite) and approximately 100µL of Tween 20 to submerge the seeds. The solution was gently agitated for 5 minutes by inversion. The tube was placed into a laminar flow, and from this point onwards, precautions were carried out to keep the seeds sterile. The diluted bleach was carefully decanted off, and the seeds were then rinsed 3-6 times with sterile dH_2O.

The seeds were then placed onto germination media within sterile jars. The germination media contained 1 L of dH_2O, 6-Benzylaminopurine (BA) (1mL of 1 mg^{-1} mL), sucrose (20g), Gamborg's B5 vitamin (1mL) (Gamborg *et al*., 1968), and Murashige and Skoog medium (4.4g) (MS) (Murashige and Skoog, 1962). Before autoclaving, the solution's pH was adjusted to 5.5 (with HCl and NaOH), and agar (8g) was added. BA and Gamborg's B5 vitamin mixture were added before use once the media had cooled to approximately 60°C.

The container containing the seeds and germination media was covered with foil for 1 week. Then the foil was removed, and the seedlings were allowed to continue to germinate and develop for another 7-10 days. Once the cotyledons were larger than 5mm, they were harvested.

The harvested cotyledons were prepared for transformation by carefully removing them from the stems with the use of sterile forceps and scalpels. They were then placed into a Petri dish containing suspension solution. The suspension media contained 1L of dH_2O, sucrose (20g), Murashige and Skoog medium (4.4g), BA (2mL at 1 mg^{-1} mL), Kinetin (0.1mg L^{-1}), Acetosyringone (0.1mM), 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.2mg L^{-1}) and Gamborg's B5 vitamin mixture (1mL L^{-1}) (Gamborg et al., 1968). Before autoclaving, the solution's pH was adjusted to 5. All ingredients other than sucrose, MS and BA were added post-autoclaving to retain their bioactivity.

While the cotyledons were submerged in the suspension solution, 2-3mm was excised from the tip and the base of each cotyledon. 30-40 cotyledons were placed abaxial side down onto a Petri dish containing preculture/coculture media with sterile filter paper on its surface. The dish was then sealed with parafilm, wrapped in foil and placed in a 20°C growth room for 1-3 days. Preculture/coculture media contained 1L of dH_2O, sucrose (30g L^{-1}), Murashige and Skoog medium (4.4g L^{-1}), BA (2 mL L^{-1}), Acetosyringone (0.1mM), 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.2mg L^{-1}), Gamborg's B5 vitamin mixture (1mL L^{-1}), and Nitsch vitamin mixture

X1000 (1mL L^{-1}) (Nitsch & Nitsch, 1969). The pH was adjusted to 5.8, then agar (8g L^{-1}) was added. All ingredients other than sucrose, MS, BA and agar were added post-autoclaving to retain their bioactivity.

2.2.9 *Agrobacterium tumefaciens* Mediated Transformation of Microtom

The transformed *A. tumefaciens* was prepared 24 hours before the cotyledons completed the preculture period. This was done by retrieving the transgenic Agrobacterium out of the -80°C freezer, and while it was still frozen, a sterile inoculating loop was used to transfer some of the glycerol into a Falcon tube containing 5mL of low salt LB broth containing rifampicin (100µg mL^{-1}) and kanamycin (50µg mL^{-1}), then incubated overnight in an incubator set to 28°C and 180 rpm. The culture was pelleted at 3000 RPM for 4 minutes. The supernatant was discarded, and the pellet was resuspended in 30mL of room-temperature suspension solution. The optical density (OD) of the solution was then measured using a spectrophotometer programmed to 600nm until an OD of 0.1-0.05 was obtained.

Once a sufficient OD was obtained, while in sterile conditions, the solution was poured into the Petri dish containing the cotyledons and preculture media. The dish was carefully sealed with parafilm and then wrapped in foil. The plate was placed into an incubator set to 28°C and 50 RPM for 25 minutes. The cotyledons were removed from the solution and placed into another dish containing wash solution. The wash solution was prepared by adding 1.4g L^{-1} of MS to dH_2O, adjusting the pH to 5 and then autoclaving it. The cotyledons were rinsed by submerging them in the wash solution and then removing excess liquid by briefly placing them onto sterile filter paper (11 µm pore size). The cotyledons were then placed onto filter paper on a new plate of preculture media (now called coculture media). The plate was then sealed, wrapped in foil and placed into a 20°C growth room for 3-5 days. After 3-5 days, the plate was unwrapped and left for another 4-7 days.

2.2.10 Micropropagation of Transformed Explants

After the completion of the coculture period, the cotyledons were transferred to plates containing a regeneration media named 2Z where they remained for 14-21 days. 2Z regeneration media was created by adding sucrose (30g L^{-1}), MS (4.4g L^{-1}), Gamborg's B5

vitamin mixture (1mL L^{-1}), Nitsch vitamin mixture X1000 (1mL L^{-1}), trans-Zeatin-riboside (2mg L^{-1}), timentin & Clavulanic Acid (TCA) (750µg L^{-1}), and kanamycin (75mg L^{-1}) to 1L of dH_2O before adjusting the pH to 5.8 and then adding agar (8g L^{-1}) before autoclaving. In a similar manner to the generation of other media and solutions previously mentioned, all hormones, vitamin mixtures, and antibiotics were added before use post-autoclaving, once the media cooled to below 60°C.

Once 14-21 days had elapsed, the cotyledons were subcultured to plates containing 1Z regeneration media. This media was prepared in the same fashion as 2Z regeneration media, with the differences being that 1 mg L^{-1} of trans-zeatin-riboside was added, the concentration of kanamycin was increased to 100mg L^{-1}, and lastly, the cotyledons were placed directly onto the media without filter paper, facilitating a barrier. The plates were then sealed once more and placed back into the growth room under the same conditions. The cotyledons remained on this media for 2 weeks before it was subcultured onto new 2Z media.

Approximately 1.5 months after transformation, large transgenic calluses form on both ends of each cotyledon. When the calluses were larger than 1cm in diameter and cell differentiation was evident (leaves and shoots were present), as shown in Fig. 2.1A, the callus was excised from the cotyledon and placed onto elongation media for 3-4 weeks (Fig. 2.1B). The calluses were subcultured onto fresh culture at 2-week intervals. Elongation media was prepared in the same manner as 1Z regeneration media, with the only difference being that the trans-Zeatin-riboside concentration was reduced to 500µg L^{-1}.

Calluses that exhibited differentiation and were larger than approximately 1.5cm in diameter were subcultured on rooting media within sterile jars, where they remained until a substantial root system developed (Fig. 2.1C/D). Rooting media was prepared similarly to elongation media; however, no trans-Zeatin-riboside was included, the kanamycin concentration was reduced to 50mg $L^{-1,}$ and lastly, Indole-3-acetic acid (IAA) was added at 1mg L^{-1}.

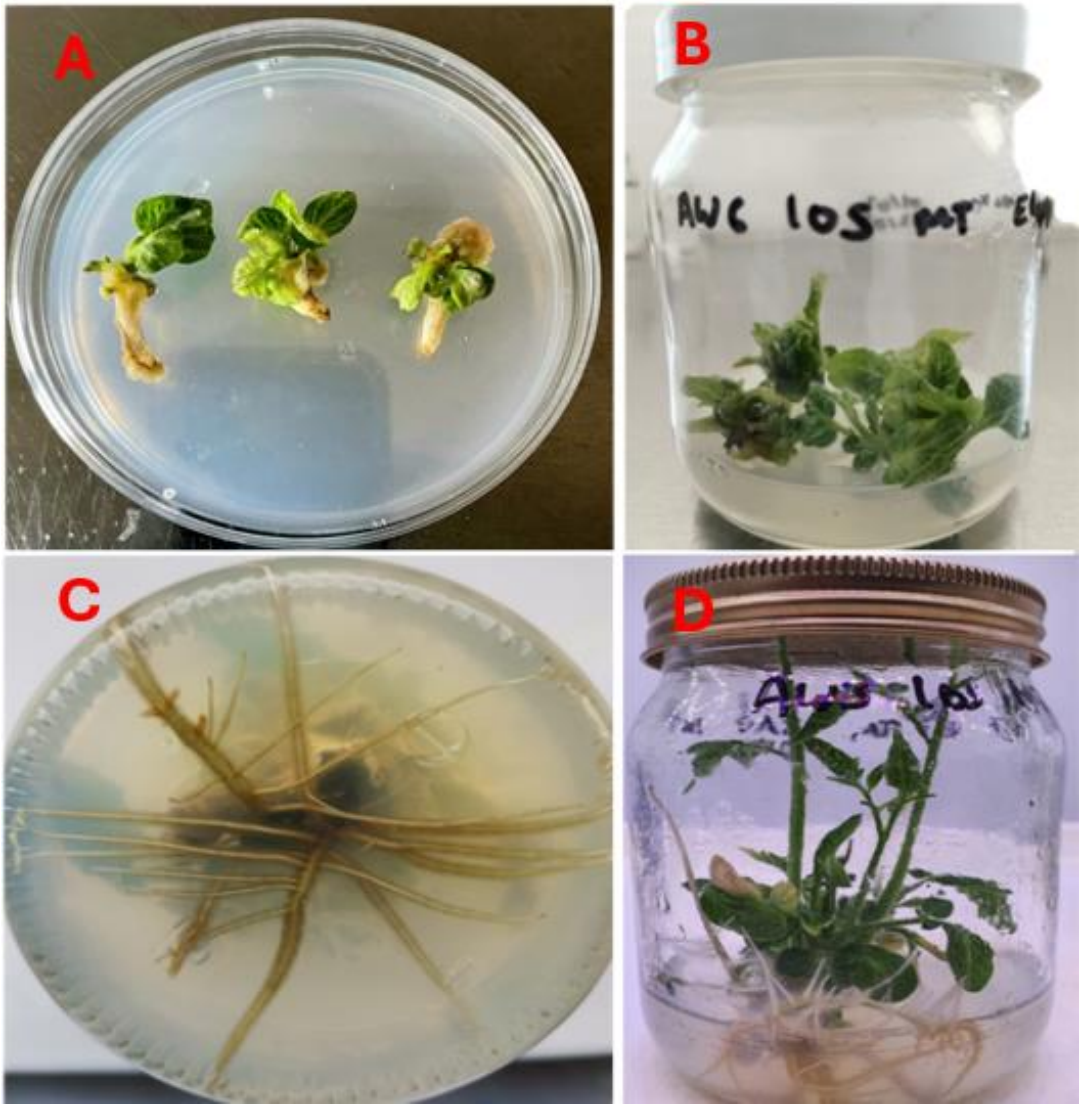

Figure 2.1. *Examples of explant stages that can be observed during micropropagation. Image* **(A)** *shows the stage at which cells within the calli have undergone organogenesis. This stage can be expected approximately 1.5 months after infection with Agrobacterium. Image* **(B)** *is a typical example of what can be observed 3 weeks after excising the calli shown in image A and culturing them on shoot elongation media to further promote shoot and leaf growth and development. Image* **(C)** *depicts typical root development that takes place once calli have experienced substantial shoot growth and are placed on rooting media. Image* **(D)** *shows the side profile of a plantlet that has undergone substantial root growth and is ready for weaning to the soil 3-4 months after initial organogenesis.*

2.2.11 Weaning Micro-propagated Explants to Soil

9cm planting pots were prepared by mixing vermiculite (Sinclair Pro medium-grain vermiculite) with Klasmann Potting & Bedding Compost in a 1:1 ratio, and then the mixture was pre-soaked until waterlogged. The plantlets with sufficient root systems were removed from their jars, and the media was rinsed off the roots as much as possible. This was conducted by filling a beaker with water and then repeatedly submerging the roots into the water while

manually dislodging any remaining agar with a thumb and forefinger. The leaves on the lower quarter of the stems were removed via pinching. Once completed, a hole was dug into the soil-vermiculite mix using the back end of forceps so that it was deep enough to house the roots and ¼ of the stem. The roots were then covered with the mix and lightly compacted around the stem. The pot was labelled and placed into a transparent plastic box. Before the box was closed, approximately 30 - 50mL of water was added to the bottom of the box to provide humidity.

The box remained closed for approximately 7-14 days before the lid was partially removed (4-5cm) to allow the humidity to decrease. After an additional 7 days, the box was slightly opened. The plants were then removed and placed on plant pot trays, and then were well watered for the following week. Once the plants had become established in the soil and new leaves had developed, the plants were watered to field capacity at 2-to-3-day intervals.

2.2.12 Growth of Plant Material

All tomato plants were grown under controlled environment conditions to ensure consistent developmental and physiological responses. Plants were cultivated in a growth chamber with a 16-hour photoperiod and a constant light intensity of 400 μmol m^{-2} s^{-1} provided by full-spectrum LED fixtures. Daytime and nighttime temperatures were maintained at 27 °C and 19 °C, respectively, with a relative humidity of 75% throughout the growth period.

Plants were grown in 1L pots containing Klasmann Potting & Bedding Compost and irrigated at 2-day intervals. CO_2 concentration was maintained at ambient levels (approximately 400–420 ppm), and no supplemental CO_2 enrichment was applied. Growth conditions were monitored daily to ensure environmental consistency across all genotypes.

2.2.13 Transformation Verification Via PCR

The presence of the transgenes was observed through PCR reactions using forward (KanF1 - AAGATGGATTGCACGCAGGTTCTCC) and reverse (KanR1 - AACGCTATGTCCTGATAGCGGTCC) primers for the kanamycin resistance gene that was present in all Level 2 constructs. The reagents included in each reaction were as follows: 12.5μL Quick-Load® Taq® 2X Master mix (New England Biolabs, Hertfordshire, UK), 0.5μL

10µM KanF1, 0.5µL 10µM KanR1, 1µL DNA sample and 10.5µL dH_2O. The thermocycler was set to the following parameters: 1 x (96°C for 2 minutes), 35 x (96°C for 15 seconds, 60°C for 15 seconds, 72°C for 90 seconds) and 1 x (72°C for 5 minutes). PCR products were run on a 1% agarose gel for 30 minutes at 145 volts. The banding patterns were compared to the 1 Kilo-base ladder to assess the size, with an expected banding pattern of 657 bp for positive results. Controls were also run on the agarose gel. The positive control was in the form of the kanamycin level 1 construct, and the negative control was DNA extracted from untransformed wild-type tissue.

2.2.14 Determining Transgene Expression Levels

2.2.14.1 qPCR

Table 2.1. *Primer sequences used for transgene expression analysis by qPCR. Forward and reverse primer sequences are listed for Arabidopsis thaliana-derived overexpression genes (SBPase, FBPa).*

Transgenic Gene Name	Forward Primer	Reverse Primer
AtSBPase (AW3)	AGACCATCATCAACCTCGAC	CCACAAATTCATAACACAACAAGCC
AtFBPA (AW4)	TGCTTCTCCCATACACATCATT	GCACACAAGCTTTTTATTTGACACA

Table 2.2. *Primer sequences used for endogenous gene expression analysis by qPCR. Forward and reverse primer sequences are listed for each target Solanum lycopersicum-derived silencing gene (iCAO, iSBPase). The primers for the housekeeping gene (Expressed (SGN-U346908)) are also*

Endogenous Gene Name	Endogenous forward	Endogenous reverse
SlCAO	TGGCCAGGAAATGATCCACC	CCTGCTTTGAGCTCCATCCT
SlSBPase	ATACACCGGAGGAATGGTGC	GCTGATCGAATACGATTGTTTCCC
Expressed	GCTAAGAACGCTGGACCTAATG	TGGGTGTGCCTTTCTGAATG

RNA was extracted from ~100 mg of frozen tomato tissue (pericarp, 25–27 DPA) using the RNeasy Plant Mini Kit (Qiagen) with on-column DNase I treatment to remove genomic DNA contamination. RNA quality and concentration were assessed by Nanodrop spectrophotometry (A260/280 ratio 1.9–2.1) and agarose gel electrophoresis to confirm integrity. 1 µg of RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the

manufacturer's instructions. The resulting cDNA was diluted 1:10 in nuclease-free water before use.

qPCR was performed on a qTower^3 84g real-time PCR thermal cyclers (Analytik Jena) using sensiMix Sybr (Bioline reagents). Each 15 µL reaction contained 7.5 µL SYBR Green master mix, 0.3 µL of each primer (10 µM; final concentration 200 nM) (Table 2.1 & 2.2), 2.5 µL diluted cDNA template, and nuclease-free water (4.4 µL). Cycling conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 seconds and 60 °C for 1 minute, with fluorescence acquisition at the end of each cycle. A melt-curve analysis from 65–95 °C was included to verify specificity. All reactions were performed in technical triplicate. Regarding negative controls, two forms were used in tandem; these included a no-template and pure nuclease-free water control. Regarding the positive controls, the primers were used with the level 2 plasmids.

Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Expression levels were normalised with the use of an endogenous housekeeping reference gene (*Expressed*), which was validated for stable expression across all samples. Data represent the mean of at least three independent biological replicates, each with three technical replicates.

Before all psychological analysis, all lines were screened via PCR for the presence of the transgene.

2.2.14.2 Copy Number Analysis

Gene copy number determination was outsourced to AttoDNA, which performed the experimental work based on DNA samples prepared in-house and submitted via postal service.

2.3 Results

2.3.1 Level 1 Assembly and Transformation of E. coli

Each level 1 (L1) Goldengate cassette contained a promoter module, a gene of interest, and a terminator. For example, as shown in Figure 2.2, the cassette contained a strong fruit-specific promoter involved in early fruit development named TFM7 (Chen *et al.*, 2016). This was

followed by the gene of interest RNAi Chlorophyll a Oxygenase. The cassette was concluded with an octopine synthase terminator. This construct allows for the expression of the gene of interest within the fruit during development. The level 1 (L1) Goldengate destination vector described by Weber *et al*. (2011) contained two DraIII digestion sites, which flanked the inserted cassette. The flanking DraIII digestion sites sanctioned predictable banding patterns after digestion and observation via gel electrophoresis to determine whether the desired assembly was successful.

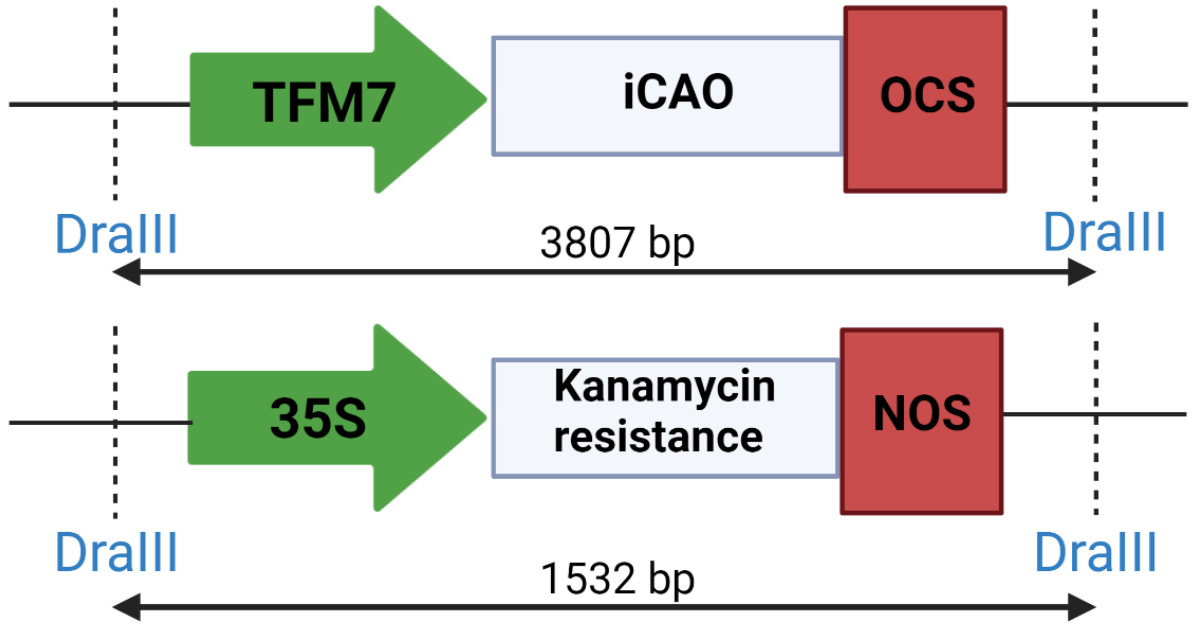

Figure 2.2. *A representative depiction of the level 1 assembled cassettes containing a promoter, gene of interest, and a terminator for the gene of interest (3807bp) and the kanamycin resistance cassette (1532bp) used as a selectable marker. The first green arrow represents the promoter TFM7, followed by the gene of interest RNAi-Chlorophyll a Oxygenase (iCAO), which in turn is followed by the strong Octopine Synthase (OCS) terminator. The second green arrow depicts the strong constitutive promoter 35S, followed by the Kanamycin resistance gene, which is followed by a Nopaline Synthase (NOS) terminator. Each cassette is flanked by a DraIII restriction site represented by the dotted line.*

The L1 constructs were used to transform chemically competent *Escherichia coli* (*E. coli*) cells. The destination vector contained a Lac operon, which caused incorrectly transformed colonies (colonies only containing empty level 1 destination vectors) to develop a blue phenotype when in the presence of X-GAL and IPTG.

2.3.2 Level 1 Plasmid Amplification, Isolation and Quantification

A minimum of 3 individual white colonies were selected and cultured before their plasmids were extracted and quantified. The isolated plasmids were digested with the DraIII restriction enzyme. Owing to the L1 destination vector containing DraIII restriction sites flanking the synthesised cassette, 2 DNA segments were produced after digestion. The larger of the segments was the destination vector, and the smaller segment was the cassette.

2.3.3 Level 2 Assembly and Transformation of E. coli

The cassettes consisted of the gene of interest, a kanamycin resistance gene (Figure 2.2), and a level 2 destination vector.

The successful transformation of the chemically competent E. coli was evident by the number of white colonies present. Red colonies were highly likely to be untransformed as the canthaxanthin biosynthesis operon remained intact after the insertion of the cassette.

2.3.4 Development and Optimisation of the Protocol

Three protocols for the transformation of tomato cotyledons were used. The first protocol used (named protocol 1) was highly inefficient as it only produced a handful of transformed plants after numerous attempts. A second protocol (protocol 2) was used to replace the inefficient and ineffective protocol 1. This second protocol was provided by the Fraser group at Royal Holloway, University of London. Protocol 2 was then further optimised and used to generate all transgenic plants used within this study. The optimised version of protocol 2 (named protocol 2.5) included numerous improvements over protocol 2, which further improved transformation efficiency.

Protocol 1 had a transformation efficiency and survival rate of approximately 2-3%, whereas protocol 2 had approximately 40-60%. Protocol 2.5 increased transformation and survival rates to approximately 75% of all cotyledons extracted (disregarding losses to contamination).

Table 2.3. *Comparison of tomato cotyledon transformation protocols and their outcomes.*

Category	Protocol 1	Protocol 2	Protocol 2.5 (Optimised)	Outcome / Effect
Overall efficiency	2–3%	40–60%	~75%	Progressive improvement
Transformation success	Very low	Moderate	High	More usable plants
Hormones	Only trans-zeatin	Added 2,4-D, kinetin, acetosyringone	Same + vitamins	Faster callus formation
Vitamins	None	None	B5 + Nitsch	Reduced necrosis
Seedling prep (BA)	No	Yes	Yes	Larger, vigorous cotyledons
light exposure	Full	Darkness	Darkness	Reduced stress
Filter paper	No	Yes	Yes	Reduced wilting
Agrobacterium strain	LBA4404	EHA105	EHA105	Better efficiency
Overgrowth	High	Minimal	Minimal	Less explant loss
Callus formation	Slow	Faster	Fast + healthy	Improved regeneration
Organogenesis	Poor	Improved	Better	Still limiting
Sucrose	20 g/L	30 g/L	30 g/L	Less nutrient stress
Kanamycin	High	Reduced	Reduced	Improved rate of development and better rooting
Subculturing	Basic	Improved	Advanced	Healthier explants
Shoot handling	Not defined	Limited	Separated	Better growth
Rooting	Poor	Improved	Optimised	Higher success
Weaning	Jar method	Improved	Vermiculite method	Higher survival
Final survival	Very low	Moderate	High	Major improvement

2.3.5 Transformation of *Agrobacterium tumefaciens*

The transformation of the EHA105 strain of *Agrobacterium tumefaciens* with the level 2 vector often yielded approximately 10-100 transgenic colonies. Post-inoculation of the agar, colonies were visible after 24-48 hours and were relatively slow growing even in optimum conditions.

Preliminarily, *Agrobacterium tumefaciens* LBA4404 was transformed and cultured in the same manner as the method described above. The same phenotypes and speed of growth were observed.

Regarding both strains, the best results were achieved when allowing the recently transformed Agrobacterium cells to incubate within the liquid media for more than the 2 hours

recommended by the protocol (section 2.1.7). The best results regarding the number of transformed colonies present were achieved when increasing the incubation time from 2 hours to 4 hours. This increased the number of colonies by approximately 50-100%.

Regarding the transformation of *E.coli* via the freeze-thaw method, it was discovered that it is best to strictly adhere to the 5 minutes at 37°C phase of the method. This is because I have observed that extending this time negatively impacts the number of transformed cells generated.

2.3.6 Generation And Preparation of Tissue for Transformation

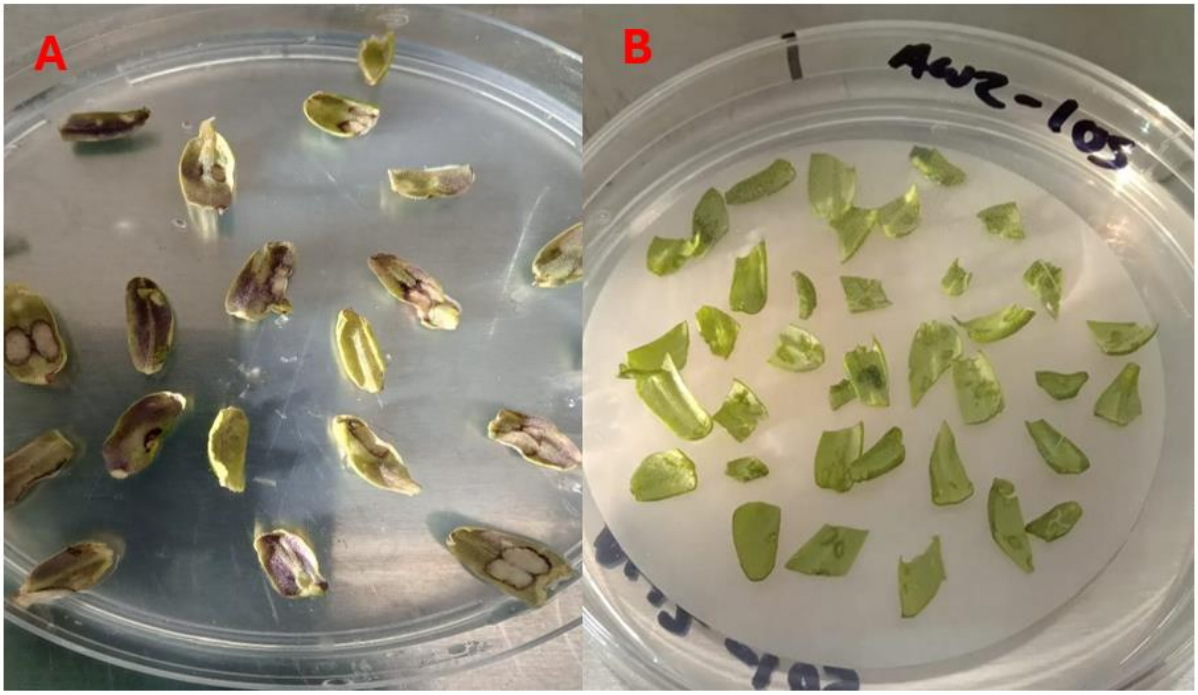

Figure 2.3. *An example of the effect of including the addition of vitamin mixtures (vitamin mixture X1000) and using filter paper to act as a barrier between the cotyledons and the media during preculture/coculture. Image* **(A)** *shows healthy tissue 1 week after inoculation. The cotyledons are not in direct contact with the media; therefore, necrosis is minimal. Image* **(B)** *shows the extent of necrosis 1 week after the cotyledons have been in direct contact with the media*

Regarding the generation of plant tissue for transformation, a key difference between the first iterations of the protocol and the subsequent protocol used was the addition of the cytokinin 6-Benzylaminopurine (BA) to the germination media. The effect of the addition of this hormone was evident when observing the phenology of the seedlings. The cotyledons produced were larger and had a visibly thicker cross-section (Panayotova, L. *et al.,* 2013). Furthermore, BA

reduced chlorophyll degradation and increased vigour and the rate of cell proliferation within the seedlings before harvest (Siddiqui *et al.*, 2011).

The preculture method used to produce the transgenic plants had similarities to the initial protocol, which was abandoned (protocol 1), but also contained differences which significantly increased transformation efficiency. These similarities included the suggested time frame in which the cotyledons should be harvested and how they should be prepared for pre-culture. The differences between the protocols involved the inclusion of hormones. Protocol 1's preculture/coculture media and suspension solution were devoid of hormones other than trans-zeatin-riboside, whereas Protocol 2 and Protocol 2.5 included the addition of 2,4-D, Kinetin, and Acetosyringone. The inclusion of these hormones allowed cotyledons to transform with protocol 2 to generate calli at a noticeably faster rate. Protocol 2.5 generated improved results over protocol 2 through the addition of Gamborg's B5 vitamin mixture and Nitsch vitamin mixture to the preculture/coculture media and suspension solution. This improved the vitality of the explants by reducing the presence of necrosis and chlorosis after a few days of remaining on preculture/coculture media.

It must be noted that Protocol 1 did not specify whether the seedlings or processed cotyledons should be protected from light by covering their containers with foil. Protocol 2 recommended that the cotyledons be uncovered only 3-4 days after the coculture period. I observed that this had the effect of increasing the vigour of the seedling and reducing signs of stress and further damage on the cotyledons after excising 2-3mm from the tip and base (Fig. 2.7B).

Another major difference between Protocol 1 and Protocol 2, which allowed Protocol 2 to produce a greater number of transformed plants, was the use of a 0.22µm filter paper. The filter paper was included to act as a barrier between the cotyledons and the preculture/coculture media, as shown in Fig. 2.7B. This resulted in fewer cases of wilting and necrosis located where the cotyledon had direct contact with the media, which occurred in Fig. 2.7A.

2.3.7 Transformation of Mictorom

The transformation efficiency for 1 protocol was extremely low (approximately below 30%). Despite some transgenic calluses being generated, in most cases, they would not survive long enough to complete organogenesis. The few calluses that did survive and undergo organogenesis either became necrotic during the 1Z regeneration media stage (see section

2.2.10) or refused to produce roots when placed into rooting media. After transforming >1000 cotyledons, only 5 plantlets survived transformation, micropropagation and rooting into the soil.

In most cases, the number of transgenic calli formed exceeded the number of usable transgenic calli. This is due to the occurrence of organogenesis being a limiting factor at this stage. It was observed that some calli generated continued to proliferate; however, they did not develop into plantlets despite the presence of cytokinin and optimal conditions. There was no obvious correlation between the instances of organogenesis not occurring and any other factors within the transformation protocol. These masses of undifferentiated cells were included in the calculation of transformation efficiency as they were an example of successful transformation. This was evident as they exhibited continuous growth in the presence of kanamycin and showed few signs of necrosis, provided they were subcultured onto fresh media, as shown in Fig. 2.8.

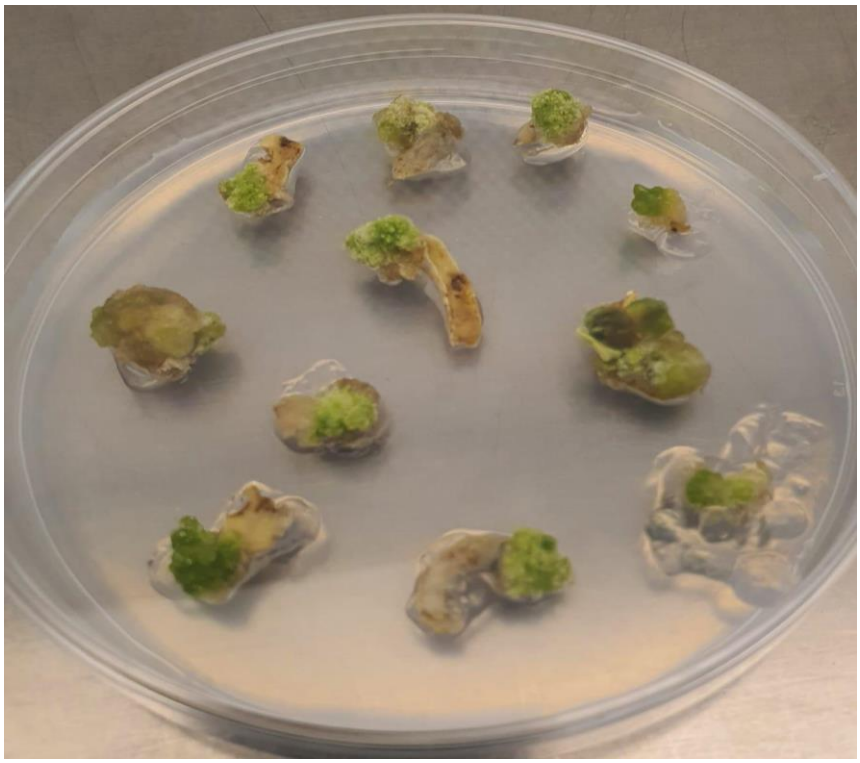

Figure 2.4. Healthy calli that have not yet undergone organogenesis while residing on regeneration media for 1.5 months.

Protocol 1 advised the use of the *A. tumefaciens* strain LBA4404. This strain has been well-documented as an effective and efficient strain for transforming tomato plants (Van Eck *et al*., 2019; Sun *et al*., 2015; Sandhya *et al*., 2022). However, as previously stated, there was much

difficulty with transformation efficiency. Furthermore, agrobacterium overgrowth during the coculture stage was a frequent occurrence, which resulted in the death of the cotyledons and plantlets involved (Fig. 2.9). *A. tumefaciens* overgrowth greatly contributed to the low transformation efficiency and post-organogenesis losses. The occurrence of *A. tumefaciens* overgrowth was greatly reduced upon the use of strain EHA105. In addition, after the use of this strain, there was a noticeable improvement in the transformation efficiency.

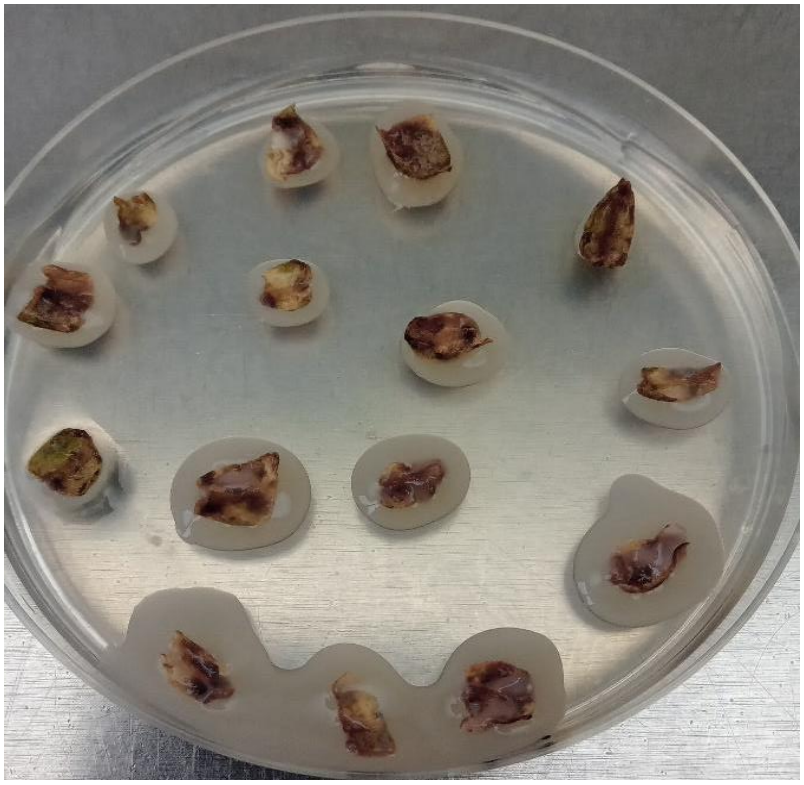

Figure 2.5. *An example of A. tumefaciens overgrowth that has resulted in severe necrosis during the co-culture stage. The cotyledons are unrecoverable at this stage.*

2.3.8 Subculturing Transformed Explants

Post-coculture, protocol 1 recommends that media used for explant regeneration (2Z and 1Z media) should contain 20g of sucrose. This was increased to 30g in protocol 2.5 to increase the sucrose available to the rapidly growing explants. The 50% increase in sucrose was accompanied by the addition of Gamborg's B5 vitamins. This improvement was made as a result of an observation made while protocol 1 was in use. This observation included many plants that survived to the regeneration stage and showed signs that could be linked to

nutritional stress, such as chlorosis of mature leaves, early flowering, leaf abscission and a reduction in growth.

Figure 2.6. *Image **(A)** depicts an example of a callus that has not had its surrounding necrotic tissue removed. Image* **(B)** *depicts the callus shown in image A after the necrotic tissue was removed to expose the living tissue. Image* **(C)** *is an example of a callus containing multiple shoots that were not separated during the regeneration phase. Image* **(D)** *shows an example of a plantlet showing signs of stress, possibly a result of a nutrient deficiency.*

An additional improvement made to the protocol involved the physical removal of any necrotic tissue present on the calli during subculturing to fresh media (Fig. 2.10A & 2.10B). This improved the overall health of the explants as well as gave the living tissue direct contact with the media. Moreover, any flowers/fruit that developed during micropropagation were excised, which encouraged the explant to focus its resources on regenerating organs such as stems, leaves and roots, which were essential to health and long-term survival. It can be said that the removal of any reproductive organs that develop allows the explants to progress more rapidly to the next stage of micropropagation.

Additionally, Protocol 1 did not provide instructions on what to do when a callus produces multiple shoots, which was a common occurrence (Fig. 2.10C). Through trial and error, it was

learned that if the shoots should be separated from one another, with as much of the callus still attached as possible, for maximum growth. This allowed for multiple duplicates of the same plant with the same transgene insertion event to exist. This observation was implemented into protocol 2.5. When the shoots were not separated and sown into the soil, they became crowded to a greater degree and competed for space and light, resulting in multiple plants with reduced health.

My revised version of protocol 2 included an improvement that involved reducing the kanamycin concentration by half to promote rooting while in rooting media. Furthermore, the original protocol 2 recommended that the plants in the rooting media should be subcultured every 14 days. In the revised protocol 2, this was omitted to prevent damage to developing roots. Lastly, 1 mL L^{-1} of Gamborg's B5 vitamin was included in the rooting media, as without it some plants became nutrient-deficient and showed signs of stress (Fig. 2.10D).

2.3.8.1 Weaning and Rooting in Soil

Figure 2.7. *An example of a Microtom plantlet in the final stages of being weaned to the soil in an open jam jar.*

Once a callus produced a sufficient root system, it was prepared to be planted in soil. This was originally done by sowing the plantlets into a jam jar filled with approximately 1/3 of well-

hydrated soil, and then the jar was sealed for 1 week. The lid was then slowly removed over several days until the jar was completely opened (Fig. 2.10). This method had low rates of success as the environment within the jar encouraged the growth of mould and algae, which negatively impacted the roots. This method was abandoned in favour of the much more successful method mentioned in section 2.2.11.

The addition of vermiculite to the soil greatly increased the soil's ability to retain moisture, thus increasing the plant's ability to recover from being removed from agar and an environment with 100% humidity. Secondly, another observed benefit of the addition of vermiculite to the soil included reducing the need for watering. This further reduced the losses of plants in the weaning stage between waterings, such as over the weekend.

2.3.9 Molecular Cloning via Golden Gate and Transformation of E. coli

The generation of specific gene sequences through the use of Golden Gate molecular cloning was highly successful and efficient, as the methodology was relatively simple, inexpensive and highly efficient in comparison to other methods available. Additionally, this method allowed the process of confirming the correct assembly and insertion into a host (E. coli) to be effective and efficient.

During the level 1 transformation of E. coli, it was often expected that very few to no colonies would exhibit the blue phenotype, which suggested that the transformation was not successful. I observed that the transformation efficiency was slightly reduced when transforming E. coli with the level 2 construct. This is likely a result of the increased physical difficulty for the larger DNA constructs to permeate through the compromised cell membranes of the chemically competent cells. This slight reduction was predicted, and to alleviate this potential problem, NEB® 10-beta Competent E. coli (Top-10) cells were used instead of the DH5α, which were made in-house. The Top-10 cells were used over DH5α as they showed higher rates of transformation.

2.3.10 Transformation Verification Via PCR

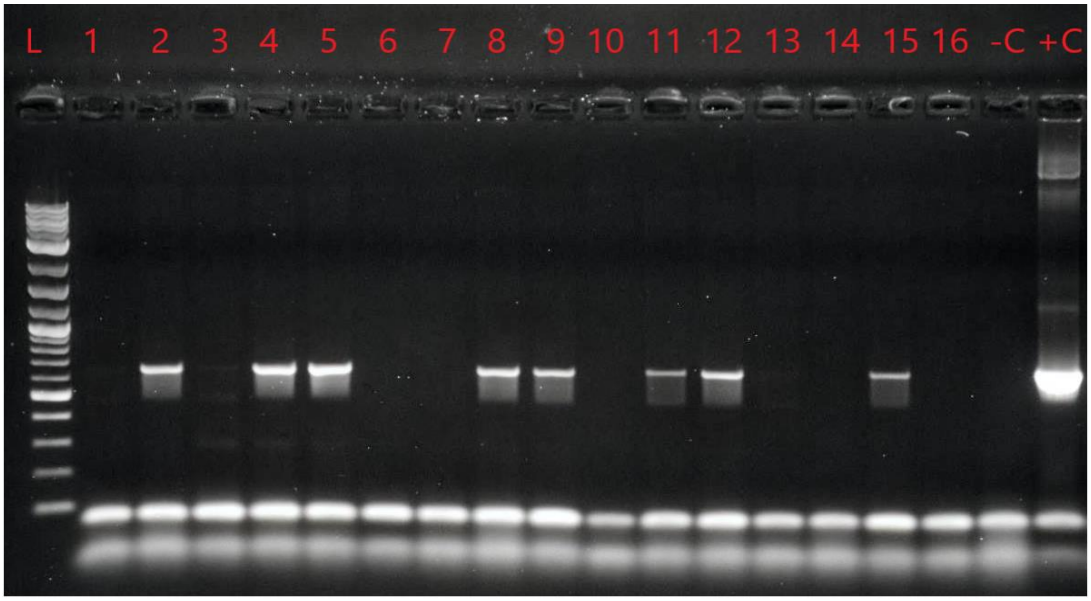

Figure 2.8. *An example of A 1% (w/v) agarose gel electrophoresis image showing amplification of the nptII (kanamycin resistance) gene from genomic DNA extracted from regenerated tomato plants. The expected product size was 657 bp. Lane L: DNA ladder (size marker); lanes 1–16: individual plant samples; lane –C: negative control (non-transformed plant DNA); lane +C: positive control (plasmid DNA containing the* nptII *gene).*

PCR was used to confirm the successful integration/presence of the transgene. PCR was performed with the use of the kanamycin resistance (*nptII*) gene as the target of amplification, which is present on all plasmid constructs used in transformation. The expected size of the amplified product was 657 base pairs (bp).

As shown in the gel electrophoresis image (Fig. 2.11), DNA was extracted from assumed transgenic plants and subjected to PCR using primers specific to the *nptII* gene. The gel demonstrates clear and discrete amplification products corresponding to the expected 657 bp in several lanes, indicating successful integration of the transgene. Specifically, lanes 1 through 16 show a mixture of positive and negative results, with distinct bands at 657 bp observed in lanes 2,4, 5, 8, 9, 11, 12, and 15. These samples can be considered undoubtedly positive for the transgene. Lanes 1 and 3 show very faint banding, which suggests the presence of the *nptII* gene, but at very low quantities. These bands may be faint as a result of the use of degraded DNA, the presence of contaminants in the sample during PCR, or a combination of poor DNA extraction efficiency and inaccurate quantification. The DNA extraction, PCR and gel electrophoresis of these samples were repeated to improve the certainty of my result.

In contrast, lanes 6, 7, 10, 13, 14, and 16 lack the bands that indicate the presence of the transgene, indicating that these samples are either non-transgenic or that the DNA quality was too low to support amplification. The lower bands at the bottom of the gel likely represent primer dimers and residual RNA, as the samples were not treated with RNase.

The negative control (lane marked "–C") shows no amplification at 657 bp, confirming that there was no contamination or nonspecific amplification. The positive control ("+C"), which consisted of the level 2 plasmid DNA containing the *nptII* gene, shows a strong single band at the correct size, validating the PCR conditions and confirming the expected product size.

Overall, these results confirm the presence of the transgene in the majority of tested samples. The presence of a single, discrete band at 657 bp in multiple lanes strongly supports the conclusion that these plants are transgenic.

2.3.11 qPCR and Copy Number Analysis

Quantitative PCR analysis conducted on RNA derived from fruit taken from the independent T_0 tomato lines (Table 2.3) showed that expression of the RNAi-CAO and RNAi-SBPase constructs successfully caused very low expression of the genes of interest. All iCAO and iSBPase lines tested showed more than a 99% decrease in expression relative to wild-type.

The Overexpression constructs SBPase and FBPA exhibited a range of transgene expression (Table 2.3). A few lines (AW3-8 & AW3-23, and AW4-7) maintained expression relatively close to WT expression levels (100%). In contrast, others exhibited strong overexpression, including one FBPA line reaching a 927% increase relative to WT expression. Overall, the majority of lines showed changes in expression levels within the fruit that were expected based on the transgenes they contained. However, AW3-41 did not show the expected change in expression, as the qPCR results (Table 2.3) showed that this line experienced a significant reduction in the quantity of SBPase transcripts.

Construct	Line	Expression relative to WT (%)	NPTII_ T0
Wild-type	WT	100	0
iCAO	AW1-5	0.841	1 to 2
iCAO	AW1-26	0.184	1
iCAO	AW1-35	0.347	1
iCAO	AW1-45	0.828	4
iCAO	AW1-57	0.048	2
iCAO	AW1-67	0.108	-
iCAO	AW1-77	0.487	-
iCAO	AW1-85	0.000	4
iSBPase	AW2-9	0.074	4
iSBPase	AW2-10	0.480	-
iSBPase	AW2-39	0.297	-
iSBPase	AW2-58	0.002	-
iSBPase	AW2-72	0.191	11
iSBPase	AW2-79	0.003	1
iSBPase	AW2-90	0.139	-
SBPase	AW3-8	51.644	-
SBPase	AW3-23	100.928	-
SBPase	AW3-30	212.874	2
SBPase	AW3-33	404.648	2
SBPase	AW3-41	0.001	-
SBPase	AW3-65	286.791	2
SBPase	AW3-87	239.496	3
SBPase	AW3-97	225.822	-
SBPase	AW3-112	520.537	2
FBPa	AW4-1	252.567	-
FBPa	AW4-7	145.397	-
FBPa	AW4-19	835.902	-
FBPa	AW4-12	237.841	-
FBPa	AW4-25	319.688	4
FBPa	AW4-49	889.709	-
FBPa	AW4-66	409.350	2
FBPa	AW4-76	503.968	2
FBPa	AW4-80	927.491	4
FBPa	AW4-96	199.538	-

Table 2.4. *Relative transgene expression levels of all independent tomato lines were determined by qPCR on fruit tissue. Expression of RNAi-CAO (iCAO), RNAi-SBPase (iSBPase), SBPase, and FBPA was measured in T_0 plants by quantitative PCR and normalised against the reference gene (Expressed). Values represent percentage change in expression relative to the wild-type control, calculated using the ΔΔCt method. Positive values indicate increased expression (overexpression lines), whereas values close to zero reflect effective knockdown (RNAi lines). Each value is the mean of three technical replicates from a single biological sample. Copy number analysis was used to determine the number of insertion events, determined by the number of neomycin phosphotransferase II (NPTII) copies in the selected T0 transgenic lines. Several lines received no NPTII results due to qPCR issues, which are denoted by a "-".*

The copy number analysis (Table 2.3) was partially successful in allowing me to make an informed decision on the selection of my transgenic lines. For the majority of lines where qPCR issues did not occur, less than 5 copies of the kanamycin resistance gene, Neomycin Phosphotransferase II (NPTII), were observed. Notably, AW1-5 received an indeterminate result with 1 to 2 copies being present. Additionally, AW2-72 showed an exceptional 11 copies of the transgene.

2.3.12 Growth of Plant Material

Figure 2.9. *Images of iSBPase line 72 fruit with lesions on its surface.*

All selected lines (SBPase, iSBPase, FBPa, and iCAO) showed no difference in the rate of emergence from the growing medium (6-9 days after sowing), development of true leaves (14-18 days after sowing), inflorescence (30-36 days after sowing), and fruit ripening (60-70 days after sowing) when compared to the WT (Azy) controls.

However, unexpected differences were observed in the fruit phenotype of an iSBPase line (line 72) when compared to wild-type azygous. This observation revealed numerous lesions on the fruit surface that extended into the inner mesocarp cell layers (Fig. 2.13). Despite this, these lesions did not affect ripening time. However, overall, no experimental construct (SBPase, iSBPase, FBPa, and iCAO) showed any consistent visual difference in fruit phenotype at any stage of development in comparison to wild-type (Fig. 2.14).

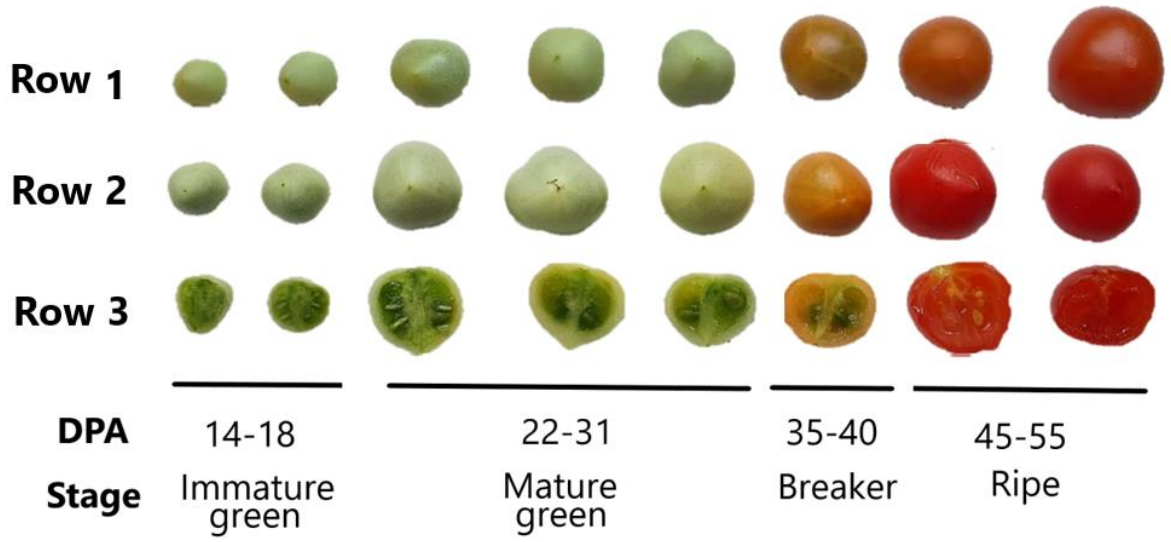

Figure 2.10. *An image showing fruit throughout the stages of development (14 to 55 DPA). Row 1 depicts fruit from the SBPase genotype, row 2 depicts wild-type fruit, and row 3 depicts the dissected cross-sections of row 2. All transgenic fruit appeared similar to wild-type throughout all developmental stages without any major changes.*

2.4 Discussion

2.4.1 Construct Design

When designing gene constructs for plant transformation, it was essential to consider how strongly, where and when the desired genes will be expressed. Thus, the choice of which promoter and terminator to use was important. They play a critical role in whether a transgene works effectively or not.

Promoters have the function of controlling when, where, and how much of a gene is expressed. Strong promoters are particularly important when high levels of expression are needed. The use of strong fruit-specific promoters, such as TMF7 and All-Flesh Fruit (AFF), was essential for my genes of interest to be expressed in high quantities throughout the fruit tissue alone. These promoters were chosen over other fruit-specific promoters as they have been well characterised in tomato and have been shown to produce stable expression throughout fruit development, specifically within the pericarp and locular gel without impacting seed development (Wang *et al.*, 2008; Liu *et al.*, 2021, 2022; Gan *et al.*, 2022; Trubanová, Shi and Schilling, 2022).

If the promoter is too weak, even a well-designed transgene may not be expressed at detectable or useful levels. On the other hand, using a strong, specific promoter ensures that the transcription machinery has a reliable and accessible binding site, which in turn increases the mRNA output. This is essential not only for detecting expression but also for ensuring that the gene product has a measurable phenotypic effect.

As mentioned, it was also essential to pick a strong terminator as they are just as important as promoters and should not be overlooked. The function of the terminator is to provide a sequence that tells the transcription machinery where to stop and help stabilise the resulting mRNA by ensuring proper polyadenylation (de Felippes and Waterhouse, 2023). A good terminator improves transcript stability by providing protection from exonucleases, prevents unwanted transcriptional read-through, and can reduce any unwanted gene silencing (Proudfoot, 2011; Nagaya *et al.*, 2010; de Felippes and Waterhouse, 2023). I selected the Nopaline Synthase (NOS), Heat Shock Protein (HSP), and Octopine Synthase (OCS) terminators for my constructs, as these can provide stable lines with long-term transgene expression and have been shown to allow for strong expression overall (Nagaya *et al.*, 2010; Gorjifard *et al.*, 2024; de Felippes and Waterhouse, 2023).

Promoters and terminators do not act in isolation, and thus the combination of both needs to be considered carefully. A strong promoter paired with a weak or leaky terminator can result in transcript instability or unintended expression. In contrast, a strong promoter-terminator pair can produce consistent and strong gene expression, often with reduced variation between transformants. In summary, selecting strong and reliable promoters and terminators was a crucial detail that gave the experiments the best chance of success.

2.4.2 RNAi vs CRISPR-Cas9

RNA interference (RNAi) was selected as the preferred method for downregulating gene expression (CAO and SBPase) in transgenic tomato plants, rather than using a CRISPR/Cas-mediated genome editing approach. This decision was guided by strategic considerations relating to partial gene suppression and functional redundancy experiments. As a result of my work aiming to assess the physiological impact of reducing, but not entirely abolishing, the expression of specific photosynthesis-related genes during fruit development, RNAi offers a valuable advantage due to its ability to achieve a partial but substantial knockdown of gene

expression. This allowed for the avoidance of the potentially lethal or severe developmental abnormalities that may occur from the complete knockout of an essential gene. In particular, when targeting genes involved in core metabolic pathways such as the CBB cycle, achieving a complete loss of function with the use of CRISPR/Cas9 is likely to result in non-viable phenotypes or confounding pleiotropic effects, complicating the interpretation of tissue-specific roles.

Another key advantage of RNAi involves its flexibility to target gene families or homologous transcripts with a similar sequence. This is particularly useful in cases when paralogous genes may compensate for one another, which can result in the knockout lines having a phenotype more akin to wild-type. The ability to design RNAi hairpin constructs that target conserved coding regions or untranslated sequences enables simultaneous silencing of multiple gene isoforms, thus providing a broader functional insight without the need for multiple, independently edited lines.

Moreover, methods involving RNAi-mediated gene silencing are also relatively well-established in tomato and other Solanaceae species. There are numerous peer-reviewed sources which provide reliable protocols for construct design, transformation, and phenotypic screening (Termolino, 2021; Leibman et al., 2021; Fuentes et al., 2016; Sun et al., 2012). The generation of stable transgenic lines expressing inverted repeat hairpin constructs can be generated and assessed with relatively high throughput and minimal need for gene-specific editing optimisation. On the other hand, CRISPR/Cas methods often require multiple guide RNA designs and extensive screening to confirm the success of gene editing, particularly when precise or homozygous mutations are required (Termolino, 2021).

While CRISPR-Cas9 systems offer the advantage of providing permanent and more thorough modifications (Belhaj *et al.*, 2015), this feature was not essential for the objectives of this study, which focused on functional characterisation. The variability and tunability of RNAi expression were better suited to experiments that required controlled alterations of gene activity. In other words, RNAi-mediated gene silencing allowed me to select multiple lines that contained a range in which the knockdown occurred. This allowed me to explore the concept of producing lines which are suited for different use cases depending on their expression level.

In summary, RNAi was chosen over CRISPR-Cas9 for its ability to achieve controlled gene silencing, its compatibility with conserved gene family targeting, and its suitability for the tissue-specific, partial knockdown experiments central to this study. While CRISPR-Cas9

represents a powerful and increasingly accessible tool for functional genomics, RNAi remains a highly effective and appropriate approach for altering gene expression in planta, particularly when subtle or reversible phenotypes are desirable.

2.4.3 Generation and Preparation of Tissue for Transformation

The method used for the preparation and generation of transgenic tissue was highly optimised through reviewing relevant literature and trial and error. The resulting method was highly efficient and effective. The importance of thoroughly sterilising the seeds and prepared media cannot be overstated, as ill preparation will cause a significant increase in post-transformation loss as a result of bacterial or fungal contamination. Contamination will further decrease the low output of successfully transformed plants as a result of explant death.

Moreover, I found that the addition of the various hormones was essential for the transformation and rapid development of the transgenic plantlets. Without the use of hormones such as 2,4-D, Kinetin, Acetosyringone, and cytokinin 6-Benzylaminopurine, the rate of successful transformation is extremely low due to the native lack of these hormones in the plant tissues. As a result of this native lack of these hormones that are beneficial to tissue culture, without the addition of these hormones to diffuse into the tissue, it would take the plant an extended time and resources to generate enough hormones for the desired event to take place. This will tax the tissues and cause an increase in necrosis.

I found that it was highly beneficial to ensure that the explants had ample access to nutrients (such as Murashige and Skoog medium, Gamborg's B5 vitamin mixture and Nitsch vitamin) required to support the rapid increase in cell differentiation and proliferation. It was also likely to somewhat aid in the explants' recovery from the A. tumefaciens infection.

Lastly, having a sufficient sucrose concentration in all of the media was imperative. When the concentration of sucrose was low ($<10g\ L^{-1}$), it resulted in the tissues having a greatly reduced rate of regeneration. This was likely due to the tissues being more dependent on their photosynthetic processes for the generation of sugars. However, it was noted that there is a limit at which the concentration of sucrose within the regeneration media becomes excessive. I found this to be above $25g\ L^{-1}$. At higher concentrations, there will be a greater amount of sugar molecules available to the regenerating tissues.

The concentration of kanamycin incorporated into the selective regeneration medium was a critical factor in optimising the selection of transformed cells expressing the Neomycin Phosphotransferase II (NPTII) gene. Kanamycin acts by inhibiting protein synthesis in non-transformed cells, while the NPTII gene confers resistance by enzymatically inactivating the antibiotic. However, I observed that increasing the kanamycin concentration above 75 mg L^{-1} significantly reduced the efficiency of regeneration. This is likely due to the NPTII protein detoxifying the antibiotic to an extent; however, higher concentrations impose excessive physiological stress even on transgenic tissue. This caused an impairment in root and shoot development. On the other hand, lowering the kanamycin concentration accelerated regeneration rates but increased the frequency of escape events (non-transformed plants that survived selection due to insufficient antibiotic pressure). Through experimental testing, I determined that 75mg L^{-1} of kanamycin was an optimal compromise as it effectively suppressed non-transgenic tissue growth while allowing for the recovery of transformed plantlets with high confidence and without excessively hindering regeneration efficiency.

2.4.4 Confirmation of Transgene Insertion

The confirmation of transgene insertion into the transgenic plants involved both PCR for the first round of plant selection, and the second and third rounds of selection for choosing transgenic lines involved qPCR and the quantification of the number of insertion events. This method was thorough and ensured that the chosen lines were, in fact, transgenic and had the desired amount of expression. As previously mentioned, the kanamycin resistance gene (NPTII) was used as a selection marker. This enabled me to ascertain the plant's transformation status. In retrospect, it may have been beneficial to incorporate an additional selection marker or reporter gene, such as Green Fluorescent Protein (GFP). This would have been beneficial, as it would have provided an increased level of certainty regarding the presence of the transgene during the initial stage of selection. Furthermore, with the use of an imager, GFP can reduce the amount of time required to genotype unconfirmed plants, as it involves an accompanying phenotype.

2.4.1 Transgene Expression

Analysis of qPCR and copy number analysis data across the generated lines shows considerable variability in expression. This highlights the importance of careful line selection and data interpretation. qPCR-based assessment of NPTII copy number in the T0 generation demonstrated a wide range of insertion events among independently transformed lines. For example, AW2-9 contained four copies, whereas AW2-72 represented a high-copy event with 11 insertions. In contrast, AW2-58 showed no detectable copies, likely due to issues with DNA quality, while the wild-type control behaved as expected with no amplification.

Despite this variation in copy number, expression analysis showed a consistent and notable reduction in transcript abundance across all transgenic iSBPase (AW2) lines relative to the wild-type baseline. This was in line with the RNA interference design of the construct, which aimed to suppress gene expression. The absence of detectable transcript accumulation, even in high-copy lines such as AW2-72, suggests that silencing was highly effective.

In contrast, overexpression lines showed a different relationship between copy number and expression. For example, line AW3-33 exhibited the highest level of transgene expression with a four-fold increase relative to the reference gene, alongside two detectable NPTII insertions. While this may suggest a contribution of multiple insertions to enhanced transcription, other lines such as AW3-30 and AW3-97 showed moderate expression (~2.1-2.2 fold). Despite this, they were still considered suitable for further study due to their stable phenotypes. Interestingly, line AW3-97 lacked detectable NPTII amplification in the T0 generation, potentially indicating a single insertion event below the detection threshold of the assay. However, its consistent expression and favourable growth characteristics justified its inclusion.

Regarding FBPa overexpression, selected lines (AW4-25, AW4-66, AW4-76, AW4-80) were chosen as they showed vigorous growth under the chosen growth conditions. As with the SPBase lines, copy number was also taken into account, as high insertion numbers have been associated with unstable expression and gene silencing in later generations. Although the selected lines contained between 2 and 4 insertions, they showed no abnormal morphologies. This suggested that these levels were acceptable for this study. qPCR analysis further confirmed that these lines achieved substantial overexpression relative to the wild type.

A similar approach was applied to the selection of iCAO lines, where three lines (AW1-45, AW1-57, AW1-85) were chosen based on a combination of high transgene expression, low copy number, and normal growth and fertility. While several lines initially appeared promising

following tissue culture, many were excluded due to infertility, likely resulting from positional effects of transgene insertion or disruption caused by excessive expression levels. These results shows the complex relationship between insertion site, copy number, and gene expression in determining overall plant performance.

As a result of the variability between independently generated lines, it became clear that interpreting results on a line-by-line basis could obscure the broader biological effects of transgene manipulation. Despite carrying identical constructs, individual lines often exhibited differing expression levels and inconsistent phenotypic responses, which complicated direct comparisons. To address this, data were pooled into two genotypic groups: transgenic and control (WT Azy). This provided a more generalised assessment of transgene effects.

Pooling allowed for normalisation of line-specific variation, thus reducing the influence of outliers. This method allowed for a clearer interpretation of the overall impact of altered gene expression. Additionally, it prevented confounding the analysis with variability as a result of individual transformation events.

It is important to acknowledge that this method represents an average response across lines with differing expression levels and does not capture the full spectrum of variation.

Overall, the combined strategy of line selection and data pooling produces a strong framework for assessing the functional impact of transgene manipulation while minimising the confounding effects of transformation-related variability.

2.4.2 Weaning Explants to Soil

I observed that the number of explants lost during the weaning-to-soil stage correlated directly with the soil's moisture levels and humidity. When soil moisture levels were low, the explants experienced extensive wilting and were unlikely to recover, resulting in death. Conversely, if the soil was waterlogged, it also led to explant mortality due to root rot and a significant increase in soil-borne insect populations. Species such as Bradysia sp. and Fungus gnats were found to prey on roots, further exacerbating root rot (Campos and McArdle, 2015).

To improve outcomes, I added vermiculite at a 1:1 ratio (per unit volume) to the soil, which enhanced its ability to retain moisture without becoming waterlogged. The inclusion of

medium-grain vermiculite likely improved soil aeration, reducing the number of explants lost during this stage and promoting additional root growth.

Humidity levels were also found to directly influence the plantlets' ability to generate new roots and adapt to changes in the substrate. As the growth room lacked humidity control, I initially managed humidity by placing the plantlets in glass jars and adjusting the moisture by unscrewing the lids. This method successfully created a humid environment, but the lack of drainage caused many plantlets to suffer from root rot. To address this issue, I developed an improved method that involved storing newly potted plants in sealable, clear plastic storage boxes. This approach effectively retained humidity while allowing for some water drainage.

The timing for each plantlet to develop sufficient roots for weaning varied by several weeks. Some plantlets grew roots more quickly than others, but the expansion of those roots to a size suitable for weaning took several additional weeks. As a result, root development and expansion proved to be unpredictable, making it challenging to determine the optimal timing for weaning accurately. Each plantlet had to be assessed individually, a process that required considerable experience gained through trial and error.

Additionally, I noted that plant size did not appear to correlate with root production capacity. However, a correlation was found between plant size and survival rate, independent of root structure. I found that plants shorter than 5 cm were less likely to survive, as it was difficult to cover their roots adequately with soil. This often led to mould infections on the lower stem and leaves due to their proximity to the moist soil.

2.4.3 Plant material

Due to time and resource restraints for experimentation, all physiological experiments mentioned in later chapters involving the CBB cycle plants were conducted using T_1 generation transgenic plants; the experiments concerning iCAO plants involved the use of T_2 generation plants. While optimal practice would otherwise be to analyse homozygous T_2 or T_3 lines to ascertain stable transgene integration and reliable expression, the project timeline necessitated the use of T_1 generation material. This decision was made to provide sufficient time for phenotypic characterisation and physiological analyses within the project's duration.

Use of T_1 plants provided several advantages. T_1 plants represent the first generation where transgene expression may be evaluated in whole, regenerated plants. These lines also generally have quite a high proportion of transgene-positive plants. This facilitates preliminary testing for transgene expression, inheritance, and overall phenotypic effects. Furthermore, using T_1 lines facilitated early screening of potentially interesting events and the determination of the lines to be taken through further generations.

It must be mentioned that there are some limitations to the use of T_1 plants. One of the major drawbacks is the genetic segregation of the transgene in this generation. As plants propagated from the transformed tissue usually carry the transgene hemizygously, the next generation (T_1) had a diversity of genotypes from homozygous through hemizygous to null segregants depending upon the number of insertion events. This heterogeneity could introduce noise to the phenotypic information and make it more difficult to interpret, particularly when quantifying the expression levels or when analysing subtle traits. Additionally, transgene expression in T_1 plants can also be heterogeneous due to position effects or silencing, and the number of insertion events is still not genetically stabilised. The observed phenotypes thus may not be indicative of those in stable, homozygous lines.

Despite these constraints, careful T_1 individual selection by molecular screening (PCR and qPCR with specific primers) was used to moderate these constraints to an extent. Additionally, the use of an appropriate sample size and replication across independent events was used to enhance the reliability of conclusions drawn from T_1 plants.

In summary, while the use of T_1 plants requires particular restrictions for transgene stability and expression variability, it allowed for an increase in the quantity and quality of physiological experiments conducted during the project. Information derived from these first-generation plants is informative and serves as a foundation for more accurate analysis in the following work on homozygous T_2 or T_3 lines in any future work.

Further information regarding plant growth conditions can be found in section 3.2.2.
Additionally, commentary on the selection of the relevant transgenic lines can be found in the "qPCR and Copy Number" section within each chapter.

2.5 Conclusion

My carefully considered choices made throughout construct design, transformation, and tissue culture allow for repeatable, accurate and reliable physiological analyses to be conducted on the generated transgenic lines. As I opted for strong fruit-specific promoters alongside appropriate terminators, this aids in ensuring that the expression is localised to the target tissues in addition to being maintained at adequate levels throughout the developmental stages of interest.

The use of RNAi over CRISPR/Cas9 provided a crucial safeguard against potential lethal or severe downstream effects, while also enabling a spectrum of gene silencing that can potentially allow for a deeper understanding of the effects. Furthermore, by optimising my methods, I minimised regeneration bottlenecks and the number of escapes (untransformed plants). This helps me to ensure that phenotypic observations accurately reflect transgene activity.

Ultimately, the work presented in this chapter provides a strong foundation by producing transgenic tomato lines for my subsequent studies aimed at understanding the role of photosynthesis in fruit.

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3 Chapter 3: Upregulating Sedoheptulose-1,7-bisphosphatase (SBPase) in Tomato Fruit to Increase Fruit Photosynthetic Rate

3.1 Introduction

Photosynthesis in green fruit has been greatly overlooked, as the contribution of photosynthetic leaves is predominantly provided by foliar photosynthesis. However, in more recent times, the topic of non-foliar photosynthesis has emerged as an area of growing interest in plant biology. In tomato (*Solanum lycopersicum*), developing fruit possesses photosynthetically active chloroplasts that are capable of light harvesting, some degree of gas exchange, and carbon assimilation during early growth stages. While fruit photosynthesis contributes only a modest fraction of whole-plant carbon gain, it has been linked to key aspects of fruit development, including growth rate, metabolite accumulation, and nutrient composition. These traits are of high interest to industry and essential to solving malnutrition in an ever-growing global population. Due to tomatoes' global economic importance as a leading horticultural crop, particularly within the fresh produce and processing industries, strategies to improve productivity by maximising underutilised photosynthetic capacity are increasingly relevant, especially in light of future challenges in agriculture posed by climate change and resource limitations.

A central enzyme within the Calvin-Benson-Bassham (CBB) cycle is Sedoheptulose-1,7-bisphosphatase (SBPase), which catalyses the dephosphorylation of Sedoheptulose-1,7-bisphosphate to Sedoheptulose-7-phosphate. This reaction is pivotal for the regeneration of Ribulose-1,5-bisphosphate (RuBP), the carbon acceptor molecule required for continued CO_2 fixation by Rubisco. Numerous studies have identified SBPase as a rate-limiting step in photosynthesis under conditions where RuBP availability constrains carbon assimilation, such as high light intensity or elevated CO_2 levels. In model species such as *Arabidopsis thaliana* and crop plants including tobacco, rice, and wheat, transgenic overexpression of SBPase has been shown to increase photosynthetic rates, improve growth, and in some cases, enhance final yield (Lefebvre et al., 2005; Driever et al., 2017; Simkin et al., 2017). These findings show that

the overexpression of SBPase could be a promising method in improving photosynthetic performance and carbon economy.

The majority of the conducted research involving SBPase has largely involved its overexpression or silencing with constitutive promoters, and foliar photosynthesis being measured (Lefebvre et al., 2005; Driever et al., 2017; Lawson et al., 2006; Gütle et al., 2016; Ding et al., 2018). Little is known about how enhancing the Calvin cycle flux might influence photosynthetic activity and downstream metabolic processes in non-foliar organs such as fruit. The photosynthetic role of fruit chloroplasts is likely to differ fundamentally from that of leaves as a result of there being a limit on atmospheric CO_2 conductance via the cuticle, in addition to there being a difference in developmental programming and sink strength. In contrast to leaves, fruit sink strength is greatly dependent on and demanding for sugars, amino acids, lipids, and pigments. Thus, the metabolic consequences of altering CBB cycle capacity in fruit cannot be assumed to mirror those observed in leaves (Hewitt, Dinar and Stevens, 1982; Paul and Foyer, 2001; Lemoine *et al.*, 2013; Nakai *et al.*, 2024).

To address this scientific knowledge gap, this study aimed to explore whether increasing SBPase activity in tomato fruit can enhance local photosynthetic efficiency and influence key aspects of fruit development and composition. SBPase-overexpressing lines were generated using a fruit-targeted promoter to drive expression specifically in developing fruit tissues (see Chapter 2, “Genetic Transformation of Microtom for full construct design and transformation protocols”. This approach allows the direct testing of the hypothesis that CBB cycle enhancement in fruit tissue will affect carbon flux, energy balance, or downstream metabolite synthesis in a physiologically meaningful way, despite the restricted rate of CO_2 uptake.

A comprehensive collection of physiological and metabolic assays was undertaken to evaluate the impact of SBPase overexpression on fruit function. These included chlorophyll fluorescence imaging to assess photosystem II efficiency, Brix measurements as a proxy for soluble sugar content, fresh weight determination, and pigment profiling to track changes in carotenoid accumulation. By examining these parameters, this study provides insights into the potential for engineering fruit-localised photosynthesis as a strategy to support or enhance fruit growth, sugar loading, and nutritional quality.

Ultimately, the work presented in this chapter aims to contextualise fruit photosynthesis not as a minor process, but as a dynamic, modifiable contributor to reproductive development and

sink strength. Understanding how the manipulation of a key CBB cycle enzyme, such as SBPase, affects these processes may inform future efforts to engineer fruit photosynthetic efficiency further and leverage both source and sink capacities to improve crop performance across a range of environments.

3.2 Materials and Methods

3.2.1 Construct Design and Generation of Plant Material

See Chapter 2 titled "Genetic Transformation of Microtom" for information regarding the design of the overexpression construct and how the transgenic tomato material was generated.

3.2.2 Experimental Design

All experiments were conducted using a minimum of three independent biological replicates (individual plants) per genotype. For each biological replicate, at least three individual fruits were sampled to account for within-plant variability and to ensure statistical robustness. Wild-type (segregated azygous lines) and transgenic lines (Calvin cycle lines were T1, and iCAO lines were T2) were screened via PCR and grown simultaneously under identical environmental conditions throughout the experimental period.

Plants were grown in two types of controlled environments: CBB plants (chapters 3,4, and 5) were grown in a Conviron A2000 reach-in growth chamber, and iCAO plants (chapter 6) were grown in a Conviron EVO-4 walk-in growth chamber, both of which held similar conditions. The conditions were a 16-hour photoperiod and a daytime light intensity of 400 μmol m^{-2} s^{-1} provided by full-spectrum LED fixtures. Daytime and nighttime temperatures were maintained at 27 °C and 19 °C, respectively, with a relative humidity of 75% throughout the growth period.

To optimise the available growth space, all plants were cultivated in 1-litre pots with Klasmann Deilmann Bedding and Potting substrate 2 soil, which allowed for a higher plant density without compromising plant health. Plant positions within the growth environment were initially randomised (complete randomisation) and subsequently rearranged every 2–3 weeks in a new random order. This approach was implemented to minimise positional bias and to

mitigate the potential impact of microenvironmental variability (e.g., differential exposure to light, temperature, or humidity) on plant growth and development.

When fruit material was harvested for physiological and biochemical analyses, fruits that were most exposed to light were selectively sampled. This was done to ensure that the data reflected fruit tissues actively engaged in light-dependent processes, such as photosynthesis, and reduced confounding effects arising from variable fruit shading or canopy position. Additionally, all fruit collected for experimentation was in the mature, unripe stage of development. More specifically, the fruits were collected 25-27 days post-anthesis (DPA) (Fig. 2.15). The 25-27 DPA mature unripe stage was specifically chosen as the primary stage of interest, as it provided fruits that were of a size that was easy to handle, as well as this stage being when the fruits are at peak chloroplast abundance and photosynthetic capacity (Piechulla et al., 1987; Lytovchenko et al., 2011). The mature unripe stage has also been shown to be the optimal stage for studying photosynthesis-driven metabolism as a result of the high metabolic activity without the significant influence of ethylene (Giovannoni, 2004).

The developmental stages were standardised across lines/plants by sowing the seeds at the same time to synchronise their growth and developmental stages as much as possible. Moreover, significant milestones such as germination and floral induction in each plant's development were noted. Upon anthesis, the stem closest to the flower was lightly marked with a permanent marker to aid in the identification of prospective fruit to collect for experimentation once 25-27 days had elapsed.

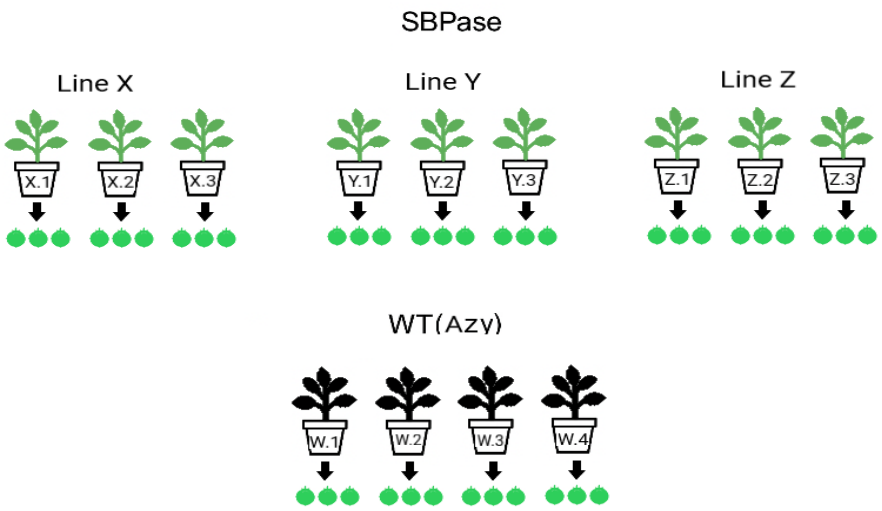

Figure 3.1. *Experimental sampling design for physiological assays in WT and SBPase tomato lines. The schematic illustrates the sampling strategy used for physiological experiments. Three independent SBPase overexpression lines (e.g. X, Y, and Z) and wild-type (Azy) plants were included in the analysis. Each line was represented by a minimum of three biological replicates (individual plants), and from each replicate, at least three mature green (25–27 DPA) fruits were harvested.*

Sampling was only conducted between 1:00 pm and 3:00 pm consistently, this was because the time of day can impact the rate of photosynthesis and the nutritional quality (Brix). Sample sizes had a few practical constraints I deemed a sample size of a minimum of 3 experimental lines as sufficient. As mentioned above and below, statistical power was increased by using 3 experimental lines derived from the same construct but different transformation events, each represented by 3 biological repeats. Sampling involved a minimum of 3 separate fruits, all at the same development stage, from all 3 biological repeats for each of the 3 experimental lines, as depicted by Fig. 3.1. All in all, a minimum of 39 fruits were used in each experiment; more samples from additional lines or biological replicates were used if they were available. Due to time and financial constraints, the experiments were conducted over one growth cycle. For all results within this chapter (and subsequent chapters), differences were considered significant at the conventional threshold for statistical significance, $p < 0.05$.

To ensure that conclusions drawn from the experiments reflect the general effect of transgene manipulation rather than line-specific variation, it was decided that data from the individual lines would be pooled for all analyses, particularly for the benefit of the chlorophyll fluorescence measurements. This decision was based on the observation of considerable variability between the independently generated transgenic lines. Although each line contained the same construct, they exhibited differing levels of transgene expression and, consequently, variable phenotypic responses. In several experiments, some lines did not consistently follow the same response patterns as the others, making it difficult to interpret the data with confidence concerning the effect of the differential expression.

Pooling the data across lines served to average the effects of these expression-level differences, reduce the impact of transgene insertion position effects, and reduce the influence of outlier behaviour produced by copy number. This approach enabled the results to be interpreted as representative of the overall impact of differential expression, rather than confusing the analysis with the effects of the individual transformation events. However, it is important to acknowledge that by pooling the data, the results represent an average response across lines exhibiting varying degrees of differential expression. As such, while the analysis remains valid

for assessing the broader effect of the gene of interest, it does not capture the full range of variation associated with different transgene expression levels.

3.2.3 Tomato Leaf DNA Extraction

Leaf tissue weighing approximately 100mg for DNA extraction was taken from newly expanded leaves once the plants had adapted and established in the soil. The tissue was then stored in 1.5 mL Eppendorf tubes and frozen in liquid nitrogen for 5 minutes. A sterile steel bead was placed into each Eppendorf tube containing the tissue before it was sealed and placed into a Geno/Grinder 2010 (SPEX SamplePrep, Cambridge, UK) programmed to run at 1500 rpm for 1.5 minutes. Once completed, the DNA extraction protocol designed and described by Edwards, Johnstone and Thompson (1991) was used.

3.2.4 Carotenoid Extraction

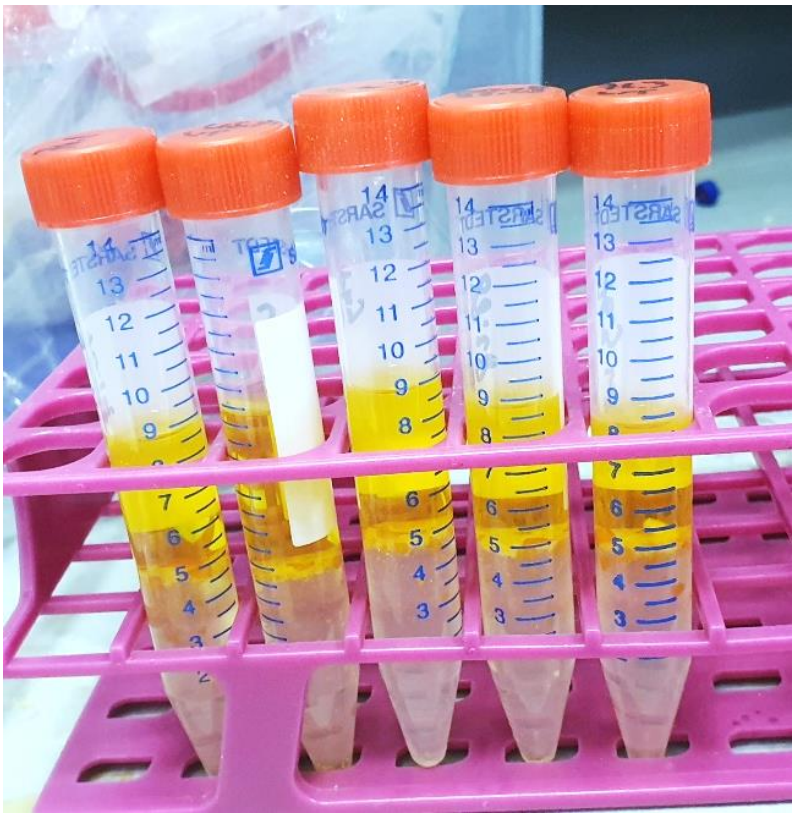

Figure 3.2. *An example of carotenoid extractions from freeze-dried ripe tomato fruit tissue with the use of hexane, acetone and ethanol. Water has been added to the extraction mixture to separate the hydrophobic hexane, which contains the carotenoids (the upper yellow fraction), from the acetone and ethanol (the lower colourless fraction).*

Carotenoid extraction assays were conducted similarly to the chlorophyll assay. Fruit was picked +10 days after ripening at approximately 55 DPA (Fig. 2.15). Once picked, the fruits were frozen at -80°C for 24 hours and then freeze-dried for 48 hours. Once dried, the fruits were individually ground to a powder with the use of pestles and mortars. 20mg was weighed, and then 8 mL of a solvent solution containing Hexane, ethanol and acetone in respective ratios of 2:1:1 was added to the powder. The falcon tubes containing the solution were inverted 3-4 times and then left within the fume hood for 30 minutes.

1.2 mL of reverse osmosis water was pipetted into the solution. The tubes were then inverted 3-4 times. Once the hexane had separated (Fig.3.2), 1.5 mL was extracted and measured spectrophotometrically at 503nm and 444nm, as these were the wavelengths used to detect lycopene and beta-carotene, respectively. The absorbance at the isosbestic wavelength of 461 nm was measured to assist in detecting other carotenoids that may interfere with the calculation of Lycopene and Beta-carotene quantities.

Overall, this method was chosen as it is highly effective and relatively quick, cheap and useful if the experimental design involves many replicates. Alternative methods that are more accurate and provide data with less variation between technical repeats could have been used, such as the use of high-pressure liquid chromatography or nuclear magnetic resonance spectroscopy. However, these methods are more expensive, labour-intensive and time-consuming. Thus, after conducting a cost-benefit analysis, I decided that conducting the assay in-house with hexane would be more efficient and economical when considering the experimental design and the number of biological and technical repeats involved.

The following equations were used to calculate the concentration of each carotenoid from the optical density (OD) measurements obtained:

The concentration of Lycopene (mg/kg) = (6.95 x Abs at 503nm – 1.59 x Abs at 444nm) 0.55 x 537 x V/W

The concentration of beta-carotene (mg/mL) = (9.38 x Abs at 444nm – 6.70 x Abs at 503nm) 0.55 x 537 x M/W

M = Mass of sample used (200mg)

W = Volume of mixed solvents added (8mL)

The equations were adapted from the works of Anthon, G., & Barrett, D. (2007).

3.2.5 Chlorophyll Fluorescence Imaging

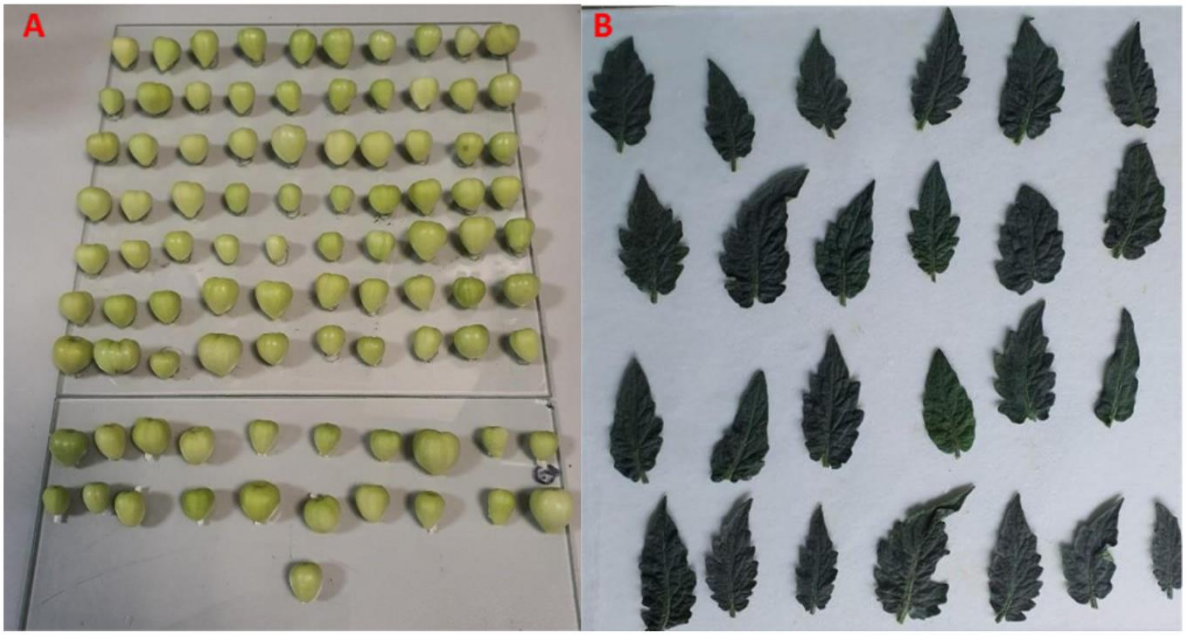

Figure 3.3. **(A)***Prepared mature unripe fruit temporarily affixed to glass plates via double-sided tape for chlorophyll fluorescent imaging light response curves after a 1-hour dark adaptation period.* **(B)** *An example of tomato leaves harvested and placed onto wet filter paper to be put into a fluorescent imager for a light response curve experiment after a 20-minute dark adaptation period.*

Chlorophyll fluorescence measurements were conducted using an fc800-d/3535-15 closed FluorCam, provided by Photon System Instruments (Brno, Czech Republic). Fruit samples were adhered to a glass plate with double-sided tape in rows of ten, while a map was created to note the fruit genotype. The fruit for analysis was chosen to be as similar in size and developmental stage as possible. Once adhered to the glass plate (Fig. 3.3A), the fruit samples were placed into the FluorCam. Methods were adapted from McAusland et al. (2019).

Chlorophyll fluorescence imaging was employed in this study as a non-invasive, high-throughput method to characterise the photosynthetic performance of fruit. This approach provided spatially resolved measurements of photosystem II (PSII) activity. This method enabled me to detect subtle changes in photosynthetic efficiency that may occur as a result of genetic modifications. Unlike gas exchange analysis, which measures net CO_2 assimilation at the whole-leaf or whole-fruit level, chlorophyll fluorescence can capture heterogeneity within a single organ, allowing direct visualisation of differences in PSII photochemistry (McAusland et al., 2019). This is particularly valuable in tomato, where chlorophyll content and photosynthetic capacity vary considerably between tissues and developmental stages,

especially in fruit that undergoes chloroplast-to-chromoplast transitions during ripening (Lado et al., 2015; Bower et al., 2020).

Parameters such as the maximum quantum efficiency of PSII (Fv′/Fm′) and the effective quantum yield (Fq′/Fm′ or ΦPSII) indicate chronic and dynamic changes in photosynthetic efficiency. The photochemical quenching coefficient (qP) was also measured; it reflects the proportion of open PSII reaction centres, while non-photochemical quenching (NPQ) measures heat dissipation of excess energy, indicating photoprotective capacity. Together, these metrics describe how light energy is allocated between photochemistry and photoprotection.

Tomato fruit structural and physiological features make direct gas exchange measurements challenging. Tomato fruit pericarp contains relatively few stomata, and these are often non-functional or exhibit limited aperture control (Blanke & Lenz, 1989; Hetherington & Woodward, 2003). As a result, internal CO_2 exchange with the atmosphere is constrained, making conventional infrared gas analysis both technically difficult and potentially unrepresentative of in situ photosynthetic dynamics. Moreover, net CO_2 assimilation measurements in fruits require custom chambers and often yield low signal-to-noise ratios due to the aforementioned minimal stomatal-mediated flux. Chlorophyll fluorescence can provide indirect evidence for changes in light energy utilisation and electron transport capacity, which are integral to driving the carbon fixation reactions likely involving recycled CO_2 from respiration and decarboxylation from the transpiration stream to be used in the CBB cycle (Baker, 2008; Murchie & Lawson, 2013; Wullschleger *et al.*, 1991; Sambo, Moorby and Milthorpe, 1977; Simkin *et al.*, 2020).

Fluorescence imaging circumvents this issue by probing the light reactions of photosynthesis independently of stomatal conductance, providing an indirect but highly sensitive means of detecting differences in photochemistry (McAusland et al., 2019).

A calibration was performed to ensure the maximum fluorescence signal was achieved. This involved increasing the far-red and white light intensity to 100% (1172 µmol m^{-2} s^{-1}), adjusting the camera shutter to 10 ms, and setting the superpulse to approximately 90% (4917 µmol m^{-2} s^{-1}) before activating it. The camera sensitivity was then adjusted until the pixel overflow error message ceased to appear, and the sensitivity was further decreased by an additional 3%.

After harvest and preparation, the fruit samples were dark-adapted for 1 hour and 24 hours (the duration is specified in each experiment) by placing them within the chamber. Leaves were dark-adapted for 20 minutes within the chamber while placed on damp filter paper (Fig. 3.3B). After dark-adaptation time had elapsed, a value of dark-adapted Fv/Fm was taken. Then, a light response curve was initiated. The protocol for the light response curve involved incrementally increasing the actinic light intensity by 5% until reaching the maximum of 1172 µmol m^{-2} s^{-1}, with measurements being recorded automatically. It took approximately 1 hour for each run, with 3 minutes at each light intensity. A saturating pulse of 0.8s was taken at the end of each light increment. Fruit chilling experiments were conducted similarly; however, the dark-adaptation period involved the fruit being stored in a dark *5°C* chiller for 12 hours beforehand.

3.2.6 Brix Assays

Figure 3.4. *Example of a fruit Brix measurement using a handheld refractometer. The scale indicates the percentage of soluble solids content (°Bx) at a reference temperature of 20 °C. The boundary between the light and dark regions corresponds to the measured Brix value; in this example, the reading is approximately 5.2 % °Bx, indicating the sugar concentration of the fruit juice sample.*

Brix (°Bx) is a measurement of the dissolved solids within a liquid. It indicates the liquid's specific gravity and is commonly used in industrial contexts to measure the dissolved sugars within a crop product, such as tomato or grape juice. In this use case, it is assumed that the majority of the dissolved solids are sugars. One degree °Bx equals 1 gram

of sucrose solute dissolved in 100 grams of solution and represents the strength of the solution as a percentage by mass (Ino *et al.*, 2023; Jaywant, Singh and Arif, 2022; Kleinhenz, 2012).

Brix measurements were conducted with the use of an analogue refractometer (Fig. 3.4). Ripe fruits were freshly picked and then cut in half. Once cut, 3 to 4 drops of juice were carefully squeezed into the glass measuring area, and then the plastic flap was closed to spread the juice over the glass evenly. The specific gravity of the juice was then measured. The equation "1 degree Brix (°Bx) = 1g of sugar per 100g of solution" was used to calculate the concentration of sucrose/glucose/fructose present.

3.2.7 Fruit Biomass Measurements

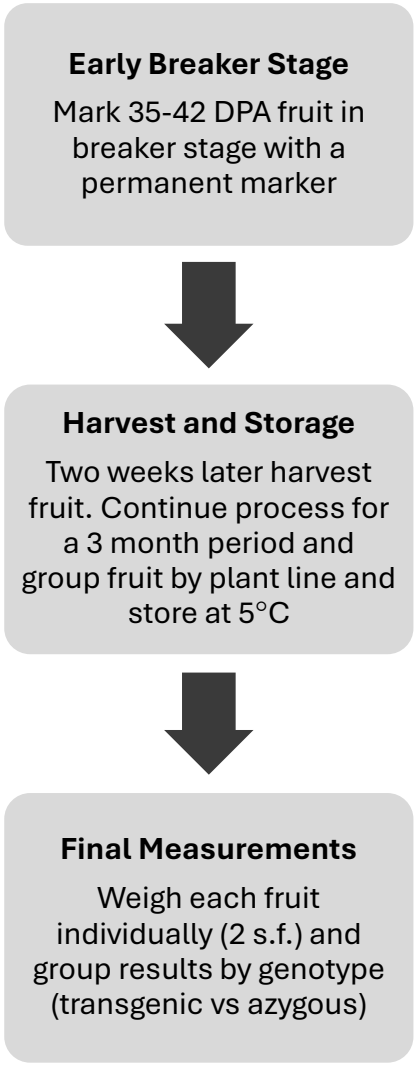

Figure 3.5. *A flow diagram showing how fruit biomass measurements were conducted.*

As described in Fig. 3.5, to ensure the fruit were fully ripe before storage, each fruit was marked at the early breaker stage (35–40 DPA (Fig. 2.15)) and harvested two weeks later. Harvested

fruit were placed in plastic bags and stored at 5 °C, grouped by plant line. After the 3-month collection period, all fruit were weighed individually to two significant figures, and the results were grouped according to genotype (SBPase and wildtype (Azy)). As a result, each fruit from the same genotype was treated as a biological replicate, providing a measure of biological variability.

3.3 Results

3.3.1 Chlorophyll Fluorescence Imaging

3.3.1.1 Leaf Tissue

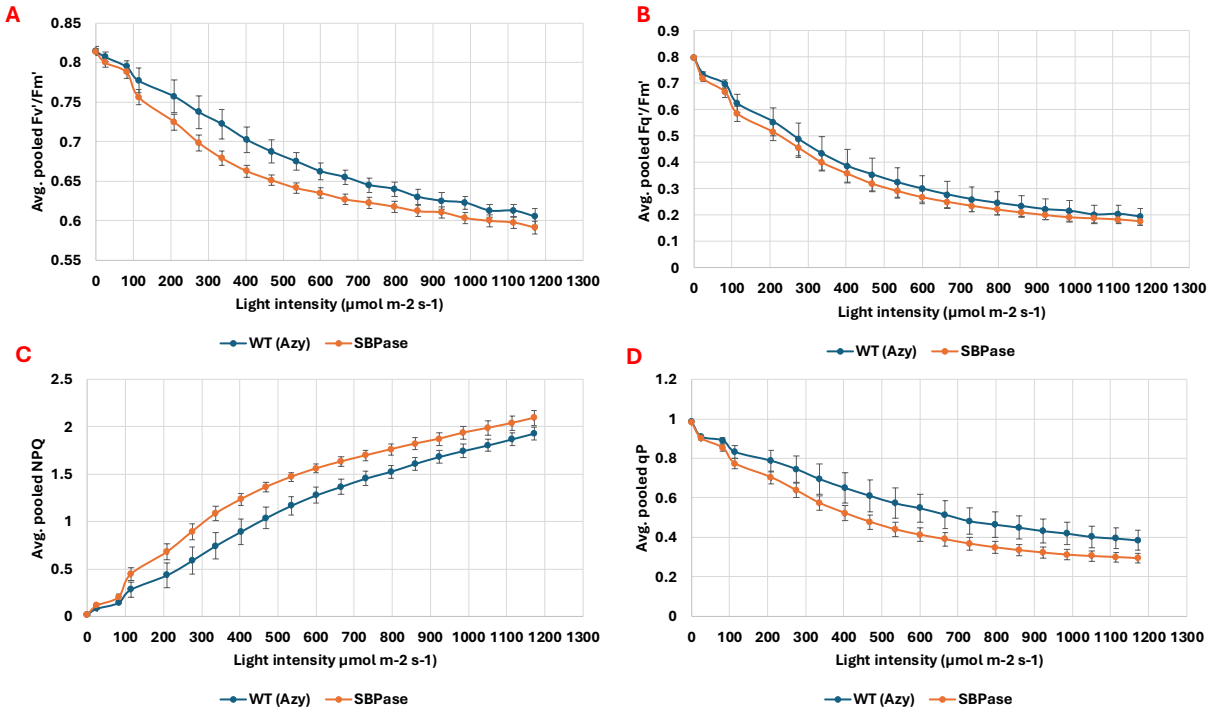

Figure 3.6. The photosynthetic performance of *young and fully expanded leaves was assessed by harvesting and dark-adapting for 20 minutes before imaging. Fruit-specific* SBPase overexpression vs wild-type (WT (Azy)). **(A)** *Fv'/Fm', the maximum quantum efficiency of PSII (the first point represents the dark-adapted value (Fv/Fm));* **(B)** *Fq'/Fm'/ ΦPSII, the effective quantum yield of PSII under light;* **(C)** *NPQ, non-photochemical quenching; and* **(D)** *qP, photochemical quenching. Results were obtained from the young and fully expanded leaves that were harvested and dark-adapted for 20 minutes before imaging. Statistical differences were observed within the Fv'/Fm' results between 403 and 535 µmol m^{-2} s^{-1} and the NPQ results between 469 and 730 µmol m^{-2} s^{-1} ($p < 0.05$). Each data point represents the mean calculated from pooled lines ($n \geq 3$). The error bars represent ± standard deviation. Blue lines indicate the WT (Azy) genotype; orange lines represent the SBPase genotype.*

In order to make a physiological comparison with fruit, leaves were measured first, using a 20-minute dark adaptation protocol that had been deemed sufficient to remove NPQ. Overall, there were some statistically supported differences between the wild-type azygous and

transgenic plant leaves in the mid-range of light intensities. More specifically, differences were observed between the maximum efficiency of PSII under light-adapted conditions (Fv'/Fm') values at and between 403 to 535 µmol m^{-2} s^{-1} ($p < 0.05$) with WT (Azy) outperforming the transgenic genotype (Fig. 3.7A). Additionally, differences were observed between the non-photochemical quenching (NPQ) values from 469 to 730 µmol m^{-2} s^{-1} ($p < 0.05$)(Fig. 3.7C), where the mutant genotype outperformed the control genotype.

As a general trend, I observed that in all tests (Fv'/Fm', the effective quantum yield of PSII (Fq'/Fm'/ΦPSII) (Fig. 3.7B), and photochemical quenching (qP) (Fig. 3.7D), the wild-type azygous genotype had consistently higher numerical values (and lower NPQ values),

3.3.1.2 1-Hour Dark Adaptation of The Fruit

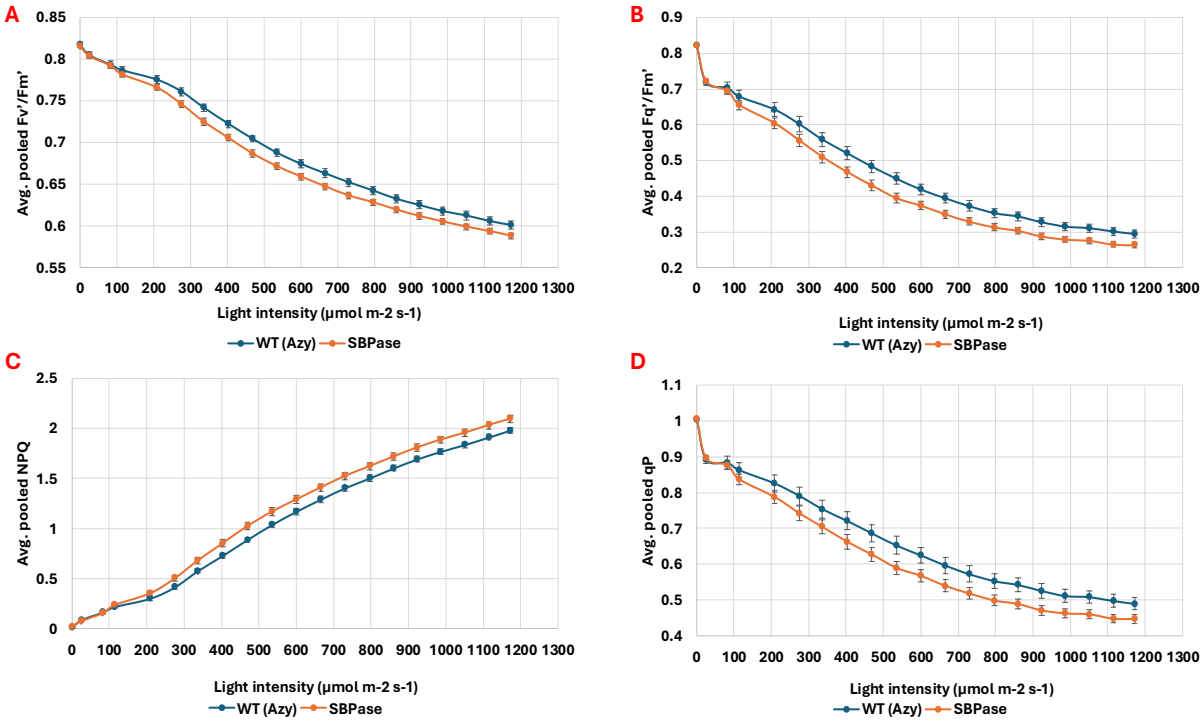

Figure 3.7. *Photosynthetic performance of SBPase overexpression vs wild-type (WT azygous) fruit under increasing light intensities following 1-hour dark adaptation.* **(A)** *Maximum quantum yield of PSII photochemistry (Fv'/Fm').* **(B)** *Effective quantum yield of PSII (Fq'/Fm').* **(C)** *Non-photochemical quenching (NPQ).* **(D)** *Photochemical quenching (qP). All parameters were measured across a light intensity gradient (0–1172 µmol m^{-2} s^{-1}) in dark-adapted fruit. Statistical differences observed within the Fv'/Fm' results (from 275 to 797 µmol m^{-2} s^{-1}), the Fq'/Fm' results (469-1172µmol m^{-2} s^{-1}), the NPQ results (275-469 µmol m^{-2} s^{-1}), and the qP results (860, 923 and 1051 µmol m^{-2} s^{-1})($p < 0.05$). Each data point represents the mean calculated from pooled biological replicates ($n \geq 3$). The error bars represent ± standard deviation. Blue lines indicate the WT (Azy) genotype; orange lines represent the SBPase genotype.*

Chlorophyll fluorescence was used to assess the effect of SBPase overexpression on PSII efficiency and photoprotective responses in fruit tissue using a 1-hour dark adaptation. I showed that the Fv'/Fm' exhibited a progressive decline at a similar rate in both genotypes (Fig. 3.8A). SBPase fruit showed a statistically significant reduction ($p < 0.05$) in Fv'/Fm' at intermediate light intensities (275–797 µmol m^{-2} s^{-1}). The Fq'/Fm' values in both the control and SBPase genotypes decreased at a similar rate in response to the increasing light intensity but were observed to contain statistically significant differences at intermediate to high light intensities (469-1172µmol m^{-2} s^{-1}) with the control genotype outperforming the transgenic (Fig. 3.8B). The NPQ results (Fig. 3.8C) showed that the SBPase fruit appeared to have a statistically significant increase in NPQ from 275 to 469 µmol m^{-2} s^{-1} ($p < 0.05$). The qP data (Fig. 3.8D) showed me that there was no consistent difference between the genotypes; however, significant differences were observed at 860, 923 and 1051 µmol m^{-2} s^{-1} ($p < 0.05$)

3.3.1.3 24-Hour Dark Adaptation of The Fruit

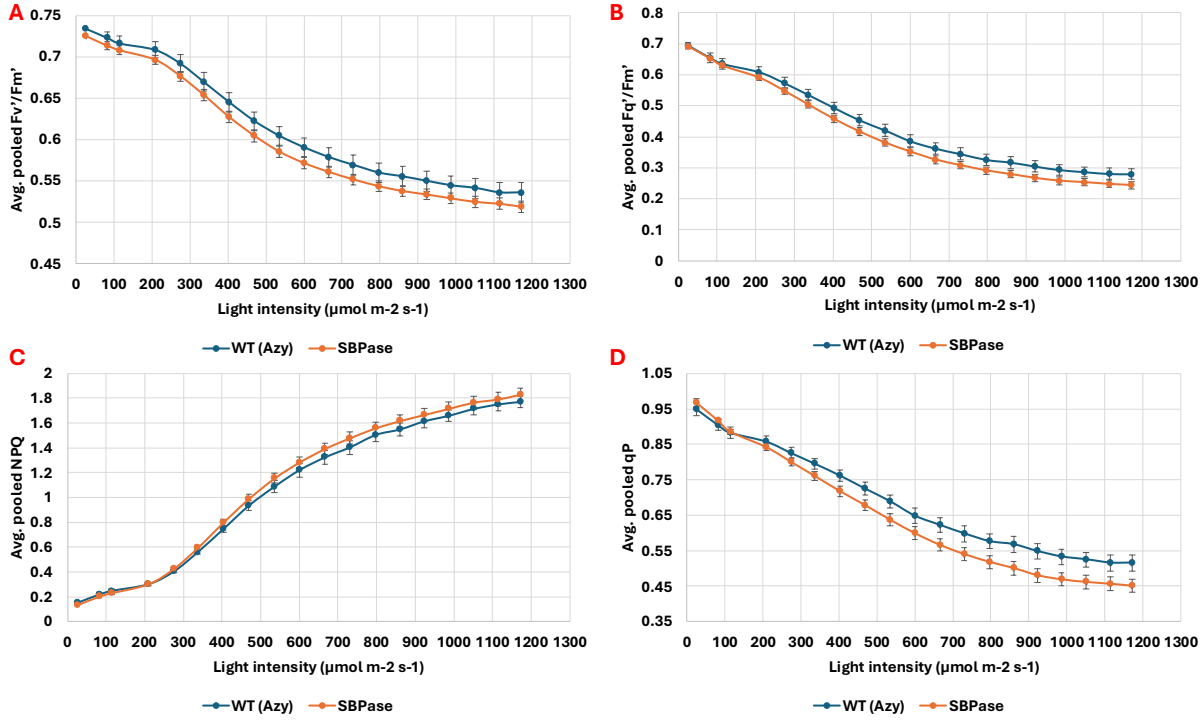

Figure 3.8. *Photosynthetic performance of wild-type and SBPase overexpression genotypes under varying light intensities following 24-hour dark adaptation of excised fruit. Chlorophyll fluorescence parameters were measured across increasing actinic light intensities (0–1172 µmol m^{-2} s^{-1}).* (**A**) *Fv'/Fm';* (**B**) *Fq'/Fm';* (**C**) *NPQ, and* (**D**) *qP. The data is plotted as the average of pooled biological replicates ($n \geq 3$) with error bars representing SD. No statistically significant differences were observed between wild-type and SBPase fruit at any light intensity or the curve as a whole for each experiment ($p > 0.05$). The first data point (at 0 µmol m^{-2} s^{-1}) for both genotypes was removed from all figures due*

to an error while recording. Blue lines indicate the WT (Azy) genotype; orange lines represent the SBPase genotype.

A longer, 24-hour dark adaptation period was used to see if this resulted in greater effects of the transgene on fruit PSII activity. The Fv'/Fm' (Fig.3.9A), Fq'/Fm'(Fig.3.9B), and NPQ (Fig.3.9C) results showed no differences at any light intensity. However, my results showed that the qP data showed significant differences at $p < 0.1$ but not $p < 0.05$ at the higher intensities (666-1172 µmol m^{-2} s^{-1}) as the p-values ranged from 0.057 to 0.09 (Fig.3.9D).

It must be noted that an error occurred at the onset of the experiment, which resulted in anomalous data at 0 µmol m^{-2} s^{-1}; thus, the data point was removed and will not be analysed. I must note that the values at 0 µmol m^{-2} s^{-1} are used to calculate NPQ; thus, the NPQ values may all be shifted for this experiment.

3.3.1.4 Fruit Photosynthetic Response to Chilling

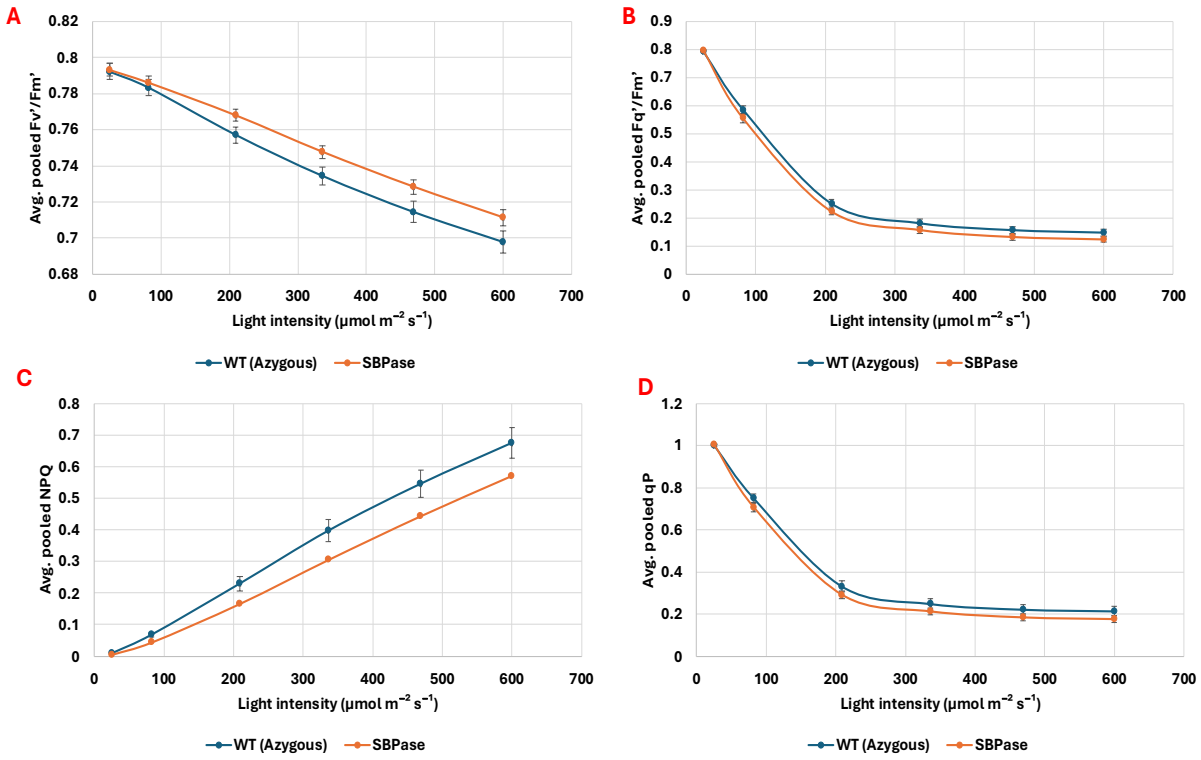

Figure 3.9. *Chlorophyll fluorescence parameters in WT (Azygous) and SBPase (SBPase overexpression) lines following 12-hour dark adaptation at 5°C to ascertain fruit chilling resistance. Four chlorophyll fluorescence parameters were measured:* **(A)** *Fv'/Fm';* **(B)** *Fq'/Fm';* **(C)** *NPQ, and* **(D)** *qP. Data is shown as mean ± SD from pooled biological replicates (n ≥ 3). Statistically significant differences in NPQ were observed between SBPase and WT (Azy) fruit between 82 µmol m^{-2} s^{-1} and 469 µmol m^{-2} s^{-1} ($p < 0.05$). Blue lines indicate the WT (Azy) genotype; orange lines represent the SBPase genotype.*

Low temperatures reduce the activity of the CBB cycle to a greater extent than electron transport. Therefore, to assess the possibility that SBPase knockdown has an impact on photosynthetic activity under a chilling spell, chlorophyll fluorescence parameters were measured in WT (Azy) and SBPase lines following 12 hours in the dark at 5°C. Fruits were then exposed to increasing actinic light intensities, and the fluorescence responses were recorded. Unpaired two-tailed t-tests showed that statistically significant differences in NPQ (Fig. 3.10C) are present between the genotypes at the light intensities between 82 µmol m^{-2} s^{-1} and 469 µmol m^{-2} s^{-1}, with the SBPase line outperforming the control. No other significant differences were observed under any parameter measured.

3.3.2 Carotenoid Content and Sugar Content by Brix Assay

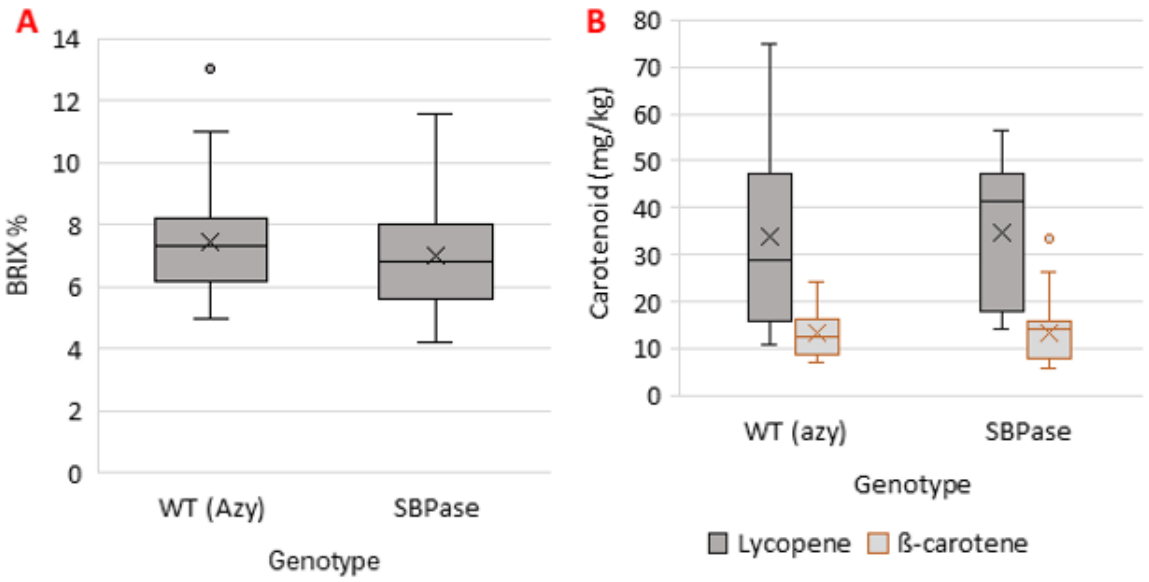

Figure 3.10. *Box and Whisker plots showing* ***(A)*** *the average Brix % ($p > 0.05$) and* ***(B)*** *a carotenoid extraction assay measured from ripe fruit from both the pooled and averaged transgenic and control WT (Azy) lines ($p > 0.05$). The central line represents the median, boxes indicate the interquartile range, whiskers show the range excluding outliers, and crosses (×) denote the mean. Outliers are represented as individual points.*

Sugar content by brix analysis was conducted on 3 selected lines containing a total of 137 fruit overexpressing SBPase and 86 WT (Azy) fruit to investigate whether overexpression of SBPase affects fruit sugar accumulation in tomato. As shown in Figure 3.11A, both genotypes exhibited a similar range and distribution of Brix values, as indicated by the overlap. More

specifically, the median °Bx was comparable between groups, as the WT (Azy) fruit contained 7.3% dissolved solids by weight and the SBPase fruit contained 6.8%. Mean °Bx values (indicated by '×' in the plot) did not differ substantially between the genotypes, as the WT (Azy) contained 7.43% and the SBPase fruit contained 6.98%. Statistical analysis using an unpaired two-tailed t-test revealed no significant difference between the WT (Azy) and the SBPase genotype ($p > 0.05$).

The carotenoid extraction assay results (Fig. 3.11B) showed that lycopene was the predominant carotenoid in both genotypes, with median concentrations of 28.8 mg/kg in WT azygous fruit and 41.3 mg/kg in SBPase fruit. The concentration of β-carotene was lower than the lycopene concentration, with median values of 13.5 mg/kg in the WT (Azy) and 13.4 mg/kg in the SBPase genotype. Although the median and upper range of lycopene concentrations appeared slightly higher in SBPase overexpressing fruit relative to WT (Azy), statistical analysis revealed no significant differences between the genotypes for either lycopene or β-carotene ($p > 0.05$). Thus, SBPase overexpression did not result in detectable alterations to carotenoid content under the conditions tested.

Therefore, my results show that overexpressing SBPase in fruit does not affect sugar content or carotenoid concentration in comparison to WT (Azy).

3.3.3 Fruit Biomass Measurements

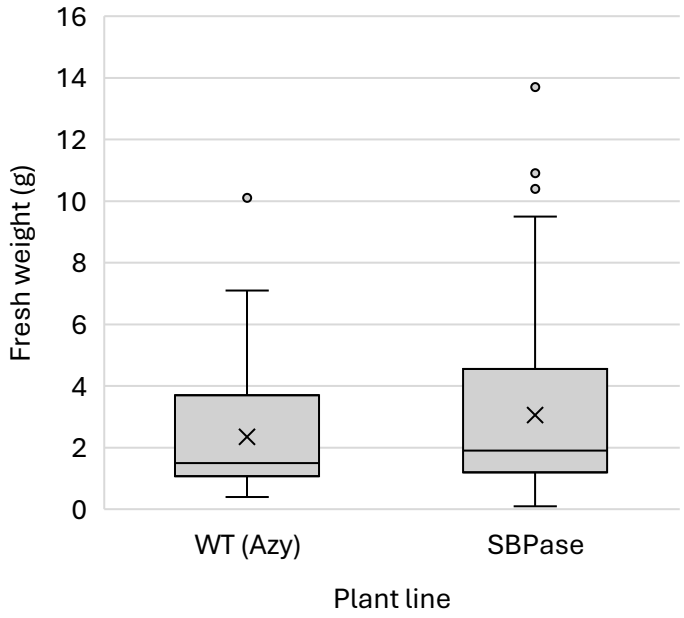

Figure 3.11. *The average fresh ripe fruit Biomass measured from WT (Azy) and SBPase. The central line represents the median, boxes indicate the interquartile range, whiskers show the range excluding outliers, and crosses (×) denote the mean. Outliers are represented as individual points (p = 0.0587).*

To evaluate the impact of SBPase overexpression on fruit development, the fresh weight of ripe fruit was measured in transgenic (SBPase) and WT (Azy) tomato lines at 3 months after the breaker stage. The distribution of fruit weights is illustrated in Fig. 3.12.

The median fruit weight was slightly higher in the SBPase lines (1.9g) compared to the WT (Azy) (1.5g). Specifically, SBPase fruits had a broader distribution, with several high-weight outliers extending above 10 g, while WT (Azy) fruits displayed a more compact range and fewer outliers. The mean fruit weight was elevated in SBPase lines at 3.1g vs 2.4g in WT (Azy), as indicated by the position of the mean marker (×) in each boxplot.

Despite the observed trend, statistical analysis using an independent Student's t-test showed that the difference in fresh fruit weight between SBPase and WT (Azy) lines was not statistically significant, though very close ($P = 0.0587$). This suggests a tendency toward increased fruit weight in SBPase-overexpressing plants, but the evidence is not strong enough to reject the null hypothesis at the $\alpha = 0.05$ threshold.

3.4 Discussion

Typically, overexpression of SBPase is anticipated to boost RuBP regeneration and thus improve Fq'/Fm' and photosynthetic performance, as shown in research focused on leaves of various species (Lefebvre et al., 2005; Rosenthal et al., 2011). However, the findings suggest that while SBPase overexpression can enhance fruit biomass under specific conditions, it surprisingly lowers Fq'/Fm', likely due to imbalances in electron transport and CBB cycle capacity, which lead to feedback limitations on photochemistry. The overexpression did not significantly affect sugar or carotenoid levels compared to the wild-type, but showed improved NPQ performance in the SBPase lines under cold stress conditions.

Fruit tissues may react differently to changes in CBB cycle enzyme levels compared to leaves. One possible explanation is that overexpression disrupts the equilibrium between electron transport and subsequent carbon metabolism, leading to feedback inhibition and decreased photochemical efficiency. This underscores the notion that the effects of SBPase manipulation

are context-dependent, with non-foliar photosynthetic tissues potentially having unique regulatory challenges.

3.4.1 Fruit-specific SBPase Overexpression Affects Both Leaf and Fruit Photosynthetic Efficiency

The presence of a leaf photosynthetic phenotype is notable, particularly given that a fruit-specific promoter was used in the construct design. Several explanations are possible. One is promoter leakiness: although the construct employed a fruit-specific promoter, such promoters can still show residual activity in other tissues (Gavrilovic et al., 2016; Estornell et al., 2009). Even slight ectopic SBPase expression could disrupt the CBB cycle coordination in leaves, particularly if unaccompanied by corresponding changes in Rubisco activation or stromal phosphate turnover (Raines, 2003; Lefebvre et al., 2005).

Another explanation is systemic feedback from altered sink metabolism in fruit. It is well established that sink demand influences photosynthetic regulation in source tissues through sucrose and trehalose-6-phosphate signalling, carbohydrate feedback inhibition, and hormonal pathways (Paul & Foyer, 2001; Lemoine et al., 2013). In tomato, modifying sink strength via invertases or sugar transporters has been shown to affect leaf photosynthetic rates and chlorophyll fluorescence profiles (Hackel et al., 2006; Xu et al., 2020).

A third possibility involves whole-plant resource allocation. The metabolic cost of transgene expression in fruit could alter nitrogen or phosphorus distribution, reducing photosynthetic protein abundance in leaves. Prior work shows that even minor shifts in nitrogen allocation can significantly affect PSII efficiency and the balance between photochemistry and photoprotection (Evans & Clarke, 2019). Developmental factors such as leaf age or specific leaf area may also contribute, though no such differences were observed in this study.

Methodological considerations must also be acknowledged. Using pointwise t-tests across irradiances increases the risk of false positives. However, the consistency of variation across multiple fluorescence parameters supports the idea that the transgenic line shows a genuine shift in PSII regulation, likely reflecting reduced ATP and NADPH consumption by the CBB cycle.

These findings gain significance when placed in the context of previous SBPase manipulation studies. Constitutive SBPase overexpression has enhanced photosynthesis and growth in tobacco, wheat, and rice (Lefebvre et al., 2005; Driever et al., 2017). In tomato, a study conducted by Ding et al. (2016) showed that the constitutive overexpression of SBPase by up to 139% increased CO_2 assimilation by approximatly 31% and total biomass by approximatly 33%. In contrast, this study specifically targeted fruit, yet still revealed systemic photosynthetic effects. This underscores the systemic consequences of organ-targeted genetic modifications. Future work involving promoter activity assays and whole-plant gas exchange could help clarify the mechanisms driving this phenotype.

The overexpression of SBPase in fruit produced distinct physiological responses, highlighting the interplay between photochemical efficiency, photoprotection, and growth. Following 1-hour dark adaptation at room temperature, photochemical efficiency declined, while NPQ rose noticeably across light intensities. NPQ is widely recognised as a protective mechanism that dissipates excess energy as heat. The stronger NPQ response in SBPase fruit suggests enhanced photoprotection, even at the cost of efficiency. This is consistent with observations that WT (Azy) tomatoes outperformed the SBPase line in Fq'/Fm' under moderate–high light, yet the SBPase genotype showed a trend leaning toward greater biomass. Given the 1:7 ratio of transgenic to wild-type plants, a larger control group might have resolved this effect more clearly.

The apparent paradox of reduced efficiency coinciding with biomass results requires careful interpretation, especially considering its near significance. No significant differences in soluble sugars or Brix were observed. If SBPase boosted carbon assimilation in fruit plastids, a rise in Brix would be expected. Its absence suggests that sugar accumulation is governed more by phloem unloading, vacuolar storage, and regulatory limits on soluble sugars than by CBB cycle activity. Since most fruit sugars originate from leaves (Hewitt, Dinar and Stevens, 1982; Falchi *et al.*, 2020), improved local fixation may instead support structural growth or respiration.

The lack of consistent qP differences at $P < 0.05$, aside from some at the higher light conditions (Fig. 3.8D), showed me that there was no consistent difference between the genotypes. This reinforces that the effects are subtle and strongly light-dependent. The predominant agreement at $p < 0.05$ between 1-hour and 24-hour dark-adapted samples strengthens the reliability of these trends, which suggest that factors beyond PSII function, such as CO_2 diffusion, sink strength, or sucrose export, may limit the impact of fruit-localised SBPase, especially at low

and intermediate light levels. Flux analyses, sugar transporter engineering, and metabolic profiling will be essential to determine whether SBPase can be combined with other strategies to enhance tomato productivity.

Chilling stress is known to inhibit CBB cycle enzymes more strongly than electron transport (Yamori et al., 2014). Fluorescence data here showed that SBPase overexpression conferred a distinct advantage under chilling, particularly in energy dissipation. This suggests enhanced CBB cycle capacity can help maintain electron use when regeneration normally becomes limiting. At low temperatures, enzymes such as SBPase and Rubisco activase are strongly inhibited, creating a carbon assimilation bottleneck. This leads to excess electron pressure and increased NPQ (Savitch et al., 2001; Haldimann & Feller, 2004; Ding et al., 2016). My findings suggest that overexpressed SBPase allowed fruit chloroplasts to use electrons more effectively, reducing NPQ activation.

Interestingly, qP declined similarly in both genotypes, indicating that differences stemmed not from PSII photochemistry itself but from downstream carbon metabolism. Thus, SBPase improved turnover capacity under chilling without substantially altering PSII redox state. These results parallel leaf studies showing SBPase enhances photosynthetic stability under stress (Ding et al., 2016; Wang, Ding & Zhang, 2020) and extend this role to fruit. This is important since non-foliar photosynthesis contributes meaningfully to fruit carbon economy and quality (Obiadalla-Ali et al., 2004; Powell et al., 2012). Enhanced resilience of fruit photosynthesis to cold could therefore benefit crops under fluctuating climates.

That said, reduced NPQ could also signal diminished photoprotective capacity if the CBB cycle becomes saturated under high light. Long-term assessments of photoinhibition, ROS accumulation, and recovery kinetics are needed to confirm whether SBPase fruit are genuinely more tolerant to chilling or at greater risk under extended stress. Subjecting whole plants to varying durations of chilling would further clarify this.

This study highlights how CBB cycle capacity helps balance productive versus dissipative energy use under stress, positioning SBPase as a key target for improving fruit resilience to temperature fluctuations.

3.4.2 Nutritional Quality is Unaffected

Previous studies have shown that increased leaf photosynthetic capacity can improve assimilate supply and potentially fruit sugar content (Lefebvre et al., 2005; Simkin et al., 2015). However, my findings indicate that fruit-localised SBPase overexpression alone does not enhance sugar accumulation. While SBPase boosts RuBP regeneration and has been linked to increased photosynthesis and biomass under constitutive expression (Driever et al., 2017; Rosenthal et al., 2011), downstream factors such as sink capacity, sugar unloading, and fruit metabolism ultimately control sugar storage (Patrick, 1997; Osorio et al., 2014).

Because fruits lack functional stomata, their primary CO_2 source is respiration rather than atmospheric uptake. Locally produced CO_2 may be reassimilated by fruit chloroplasts, but concentrations are likely much lower than those available to leaves. This limitation constrains the quantitative contribution of fruit photosynthesis to sugar accumulation, even when photosynthetic enzymes are overexpressed. It is also possible that additional carbon fixed by fruit photosynthesis is redirected to vegetative tissues via apoplastic unloading (Chen et al., 2017; Zhang et al., 2022). Environmental and developmental variables such as fruit load, light, or developmental stage, may further modulate sink responses (Ho, 1996; Xu et al., 2022). The wide variation in Brix values across both genotypes suggests high biological and environmental variability that could obscure subtle genotypic effects.

In addition to soluble sugars, carotenoid levels were also unchanged in SBPase-overexpressing fruit. This outcome is unsurprising, as carotenoid accumulation is largely governed by the regulation of plastid differentiation and the expression of biosynthetic enzymes during ripening, rather than by the immediate supply of carbon from photosynthesis (Fraser & Bramley, 2004; Sun et al., 2018). While enhanced CBB cycle activity could, in principle, provide more precursors for isoprenoid synthesis, the pathway is strongly developmentally programmed and subject to metabolic feedback control. Thus, altering carbon fixation alone is unlikely to shift carotenoid pools without concurrent changes in plastid biogenesis or carotenoid pathway regulation.

In conclusion, fruit-localised SBPase overexpression has the theoretical capacity to enhance sugar loading but did not significantly alter sugar content under the conditions tested. This emphasises the complexity of source-sink relationships and suggests coordinated modification of both assimilation and sink traits is required to shift quality attributes such as sweetness

(Hackel et al., 2006; Lytovchenko et al., 2007). The lack of significant changes in Brix or PSII efficiency reinforces that fruit-localised photosynthesis contributes little to yield-related traits. Achieving meaningful gains will likely require systemic enhancement of whole-plant source capacity, sink strength, and carbon transport, rather than relying on single-enzyme overexpression within fruit (Li, Heuvelink & Marcelis, 2015; Chang & Zhu, 2017; Burnett et al., 2016).

3.5 Conclusion

This chapter explored how the fruit-specific overexpression of SBPase affects the physiology, photochemistry, and compositional characteristics of tomatoes. I selected three transgenic lines (30, 33, and 97), which demonstrated over 100% increases in SBPase transcript levels and a low NPTII copy number, to ensure a thorough analysis. Contrary to findings from leaf studies, the increased SBPase expression in fruit did not lead to significant changes in carotenoid levels or soluble sugar accumulation. This suggests that the metabolic processes in ripening fruit are influenced by factors beyond RuBP regeneration. Additionally, the increase in fruit biomass was minimal, indicating a mild positive effect on yield.

The most notable outcomes were found through chlorophyll fluorescence analysis. In fruit that had been dark-adapted for 1 hour and 24 hours, SBPase overexpression consistently resulted in reduced Fq'/Fm' and Fv'/Fm' ratios at moderate to high light intensities. This implies that while the Calvin cycle's capacity may increase, it can also result in a photochemical cost in non-leaf tissues. Elevated NPQ responses further supported this observation, suggesting improved energy dissipation as heat to manage excess excitation pressure. Cold-stress tests reinforced this trend, showing that SBPase-expressing lines outperformed controls in NPQ but exhibited lower photochemical efficiency. Collectively, these findings indicate that overexpression of SBPase creates an imbalance between electron transport and subsequent carbon assimilation in fruit, leading to decreased photochemical efficiency despite higher enzyme levels.

These results are surprising, especially given previous evidence that SBPase overexpression enhances photosynthesis and biomass in leaves. This research illustrates that fruit photosynthesis operates under specific regulatory constraints, meaning that manipulating CBB cycle enzymes does not necessarily lead to greater productivity. Instead, SBPase may play a

key role in balancing energy capture, dissipation, and metabolic needs, which can reduce Fq'/Fm' while possibly supporting fruit biomass modestly.

Overall, this work emphasises that the significance of fruit photosynthesis cannot simply be inferred from leaf studies. The overexpression of SBPase in tomato fruit reveals a complex regulatory aspect that can lead to reduced photochemical activity even while compositional traits remain stable. These findings underline that non-foliar photosynthesis functions under distinct metabolic and biophysical pressures. Understanding the mechanisms driving these counterintuitive results will be crucial for determining whether such effects can be harnessed or managed in agriculture. With further insight, targeted alterations in fruit photosynthesis might provide new strategies for enhancing crop yield and nutritional quality in the field.

3.6 Bibliography

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4 Chapter 4: Silencing Sedoheptulose-1,7-bisphosphatase (SBPase) in Tomato Fruit Via RNAi to Determine Its Contribution to Photosynthetic Rate and Nutritional Quality

4.1 Introduction

Photosynthesis is the fundamental biological process that drives nearly all life on Earth. It involves the conversion of light energy into chemical energy and forms the primary source of organic carbon for countless ecosystems. While the majority of research has focused on photosynthesis within foliar tissues, there is increasing evidence that non-foliar organs such as stems, seeds, and fruit can also possess the ability to photosynthesise at some level. However, the physiological roles and regulatory mechanisms of photosynthesis in these non-foliar tissues remain poorly understood and characterised despite their potential to contribute to organ development, carbon economy, and sink strength.

In *Solanum lycopersicum* (Tomato), fruit photosynthesises during the fruit's early stages of development have been shown to contribute to the overall fruit metabolism, growth, and quality (Lytovchenko et al., 2011). Reseach involving this is particularly important given that tomatoes are one of the most widely cultivated horticultural crops worldwide, with significant economic and nutritional importance. Enhancing our knowledge of photosynthesis beyond leaves could provide new strategies for improving crop productivity, particularly in the context of climate change and increasing global food demand.

Sedoheptulose-1,7-bisphosphatase (SBPase) is a key enzyme of the Calvin-Benson-Bassham (CBB) cycle that catalyses the irreversible dephosphorylation of sedoheptulose-1,7-bisphosphate to sedoheptulose-7-phosphate, an essential step in the regeneration of the primary carbon acceptor molecule in photosynthetic carbon fixation, ribulose-1,5-bisphosphate (RuBP) (Raines, 2003; Raines, 2011). SBPase activity has been identified as a major control point in photosynthesis, particularly under conditions where RuBP regeneration limits the rate of

carbon assimilation, such as under high light or elevated atmospheric CO_2 (Stitt et al., 2010). Transgenic studies in model and crop species, such as *Arabidopsis thaliana*, *Nicotiana tabacum*, rice, and wheat, have demonstrated that overexpression of SBPase enhances photosynthetic efficiency, biomass accumulation, and, in some cases, yield (Lefebvre et al., 2005; Rosenthal et al., 2011; Driever et al., 2017; Simkin et al., 2017). These findings suggest that SBPase constitutes a significant biochemical bottleneck within the CBB cycle. However, the extent to which SBPase limits photosynthesis can be highly context dependent. This is because it is influenced by environmental conditions, species-specific metabolic architecture, developmental stage, and the balance between source and sink capacity (Raines, 2011; Simkin et al., 2019). SBPase is also subject to redox regulation via the ferredoxin-thioredoxin system, allowing its activation in the light and integration into chloroplast redox signalling networks (Gütle *et al.*, 2016). These can add further layers of complexity to its role in dynamic photosynthetic responses. While its function has been extensively studied in foliar tissues, much less is known about its contribution in non-foliar organs such as fruit or seeds, where the regulation and significance of the CBB cycle may differ greatly.

The targeted silencing of SBPase with the use of RNAi in tomato fruit will provide a novel strategy for exploring the metabolic role of plastid-localised photosynthesis and its effect on reproductive development. While tomato fruit possesses the ability to photosynthesise and thus generate photoassimilates in-house, the extent to which SBPase activity contributes to this remains unclear. Disabling SBPase allows for the creation of a precise molecular block within the CBB cycle; this is hypothesised to manifest as a near-complete reduction in fruit photosynthetic efficiency in addition to a significant decrease in sugar and biomass accumulation.

This study enables the determination of whether localised carbon assimilation is functionally significant or simply a residual feature of chloroplast maintenance. Such a loss-of-function approach will enable the investigation into how the absence of CBB cycle flux impacts plastidial energy balance, carbon recycling, and downstream biosynthetic pathways in fruit tissues. Importantly, it will aid in uncovering whether developing fruit can compensate metabolically through increased import of photoassimilates or reconfiguration of plastid function. This experiment offers a valuable opportunity to distinguish the metabolic roles of fruit chloroplasts from those in foliage, providing new insights into source-sink interactions,

tissue-specific regulation of photosynthesis, and the broader metabolic network supporting fruit development.

To explore the metabolic role of fruit-localised photosynthesis in tomato, RNAi-SBPase (iSBPase) tomato fruit were produced (see in chapter 2 for details on their production). Experimental methods, including chlorophyll fluorescence, sugar content analysis via Brix, fruit weight analysis, and carotenoid content analysis, were conducted to ascertain the effect of silencing SBPase on fruit development, quality and carbon partitioning.

4.2 Materials and Methods

The construct design and the growth conditions for the plant material can be found collated in the 2 chapter. The full physiology methods can be found in Chapter 3. For all results in this chapter, differences were considered significant at $p < 0.05$.

4.3 Results

4.3.1 Chlorophyll Fluorescence Imaging

4.3.1.1 Leaf Tissue

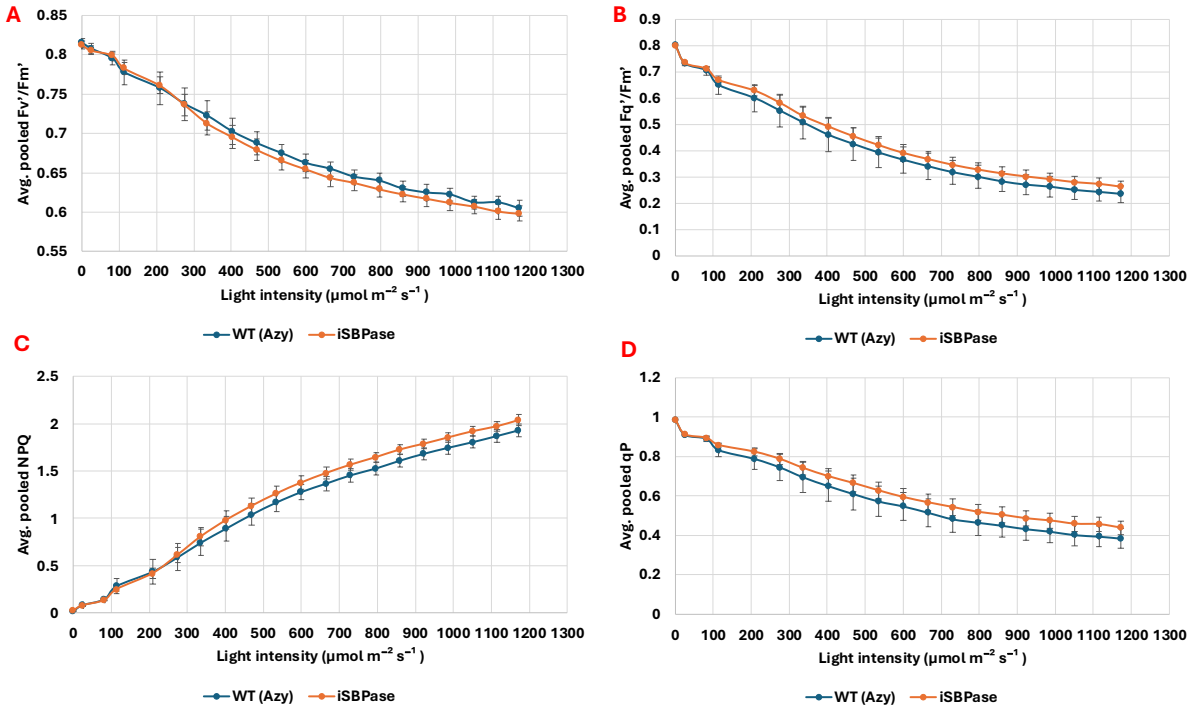

Figure 4.1. *A leaf fluorescence light response curve showing the* **(A)** *Fv'/Fm',* **(B)** *Fq'/Fm' /ΦPSII,* **(C)** *NPQ, and* **(D)** *qP. Results were obtained from the young, fully expanded leaves that were harvested and*

dark-adapted for 20 minutes before imaging. Error bars represent standard deviation. Blue lines indicate WT (Azy) controls; orange lines represent the iSBPase genotype.

To investigate whether the knockdown of SBPase with the use of a fruit-specific promoter had an impact on the photochemical efficiency of leaf photosystem II (PSII), chlorophyll fluorescence light response curves were conducted on young, fully expanded leaves of iSBPase and wild-type azygous tomato plants that were 6 weeks post-germination. Leaves were dark-adapted for 20 minutes before measurements and subjected to increasing levels of actinic light to assess key photosynthetic parameters: maximum quantum efficiency of PSII in a dark-adapted state (Fv/Fm) (Fig. 4.2A), maximum quantum efficiency of PSII in the light (Fv'/Fm') (Fig. 4.2A), effective quantum yield (ΦPSII, or Fq'/Fm') (Fig. 4.2B), non-photochemical quenching (NPQ) (Fig. 4.2C), and photochemical quenching (qP) (Fig. 4.2D).

Dark adaptation is used in fluorescence measurements to remove non-photochemical quenching processes and assess other photosynthetic parameters from an initial relaxed state. Whilst the dark-adapted value of Fv/Fm represents the maximum quantum efficiency of PSII, the Fv'/Fm' represents the maximum possible efficiency of PSII under prevailing light conditions, when all reaction centres are open and regulatory mechanisms such as non-photochemical quenching, state transitions, and energy-dependent quenching are active. By definition, this differs from Fv/Fm (shown as the data point at 0 μmol m^{-2} s^{-1} in all Fv'/Fm' graphs presented in this study) as Fv/Fm represents the maximum potential quantum efficiency of PSII photochemistry when all centres are open during a dark-adapted state, with no regulatory quenching active. The Fq'/Fm' is the actual operating efficiency of PSII in the light and can be used to calculate in situ electron transport through PSII (Murchie and Lawson 2013).

As mentioned, NPQ and qP were also measured; these indicate different aspects of the current usage of the absorbed light energy. NPQ reflects the rate at which constant energy is dissipated as heat rather than being used for photochemistry. In other words, it represents the controlled photoprotection mechanisms present to safely dissipate excess excitation energy as heat in the light-harvesting complexes (LHCII). This differs from qP as it represents the fraction of open PSII reaction centres that are capable of using absorbed light energy for photochemistry.

Across all measured parameters, iSBPase leaves exhibited similar responses to increasing light intensity when compared to wild-type azygous controls; no significant differences were observed at any intensity within all measurements (Figure 4.2).

4.3.1.2 1-Hour Dark Adaptation of The Fruit

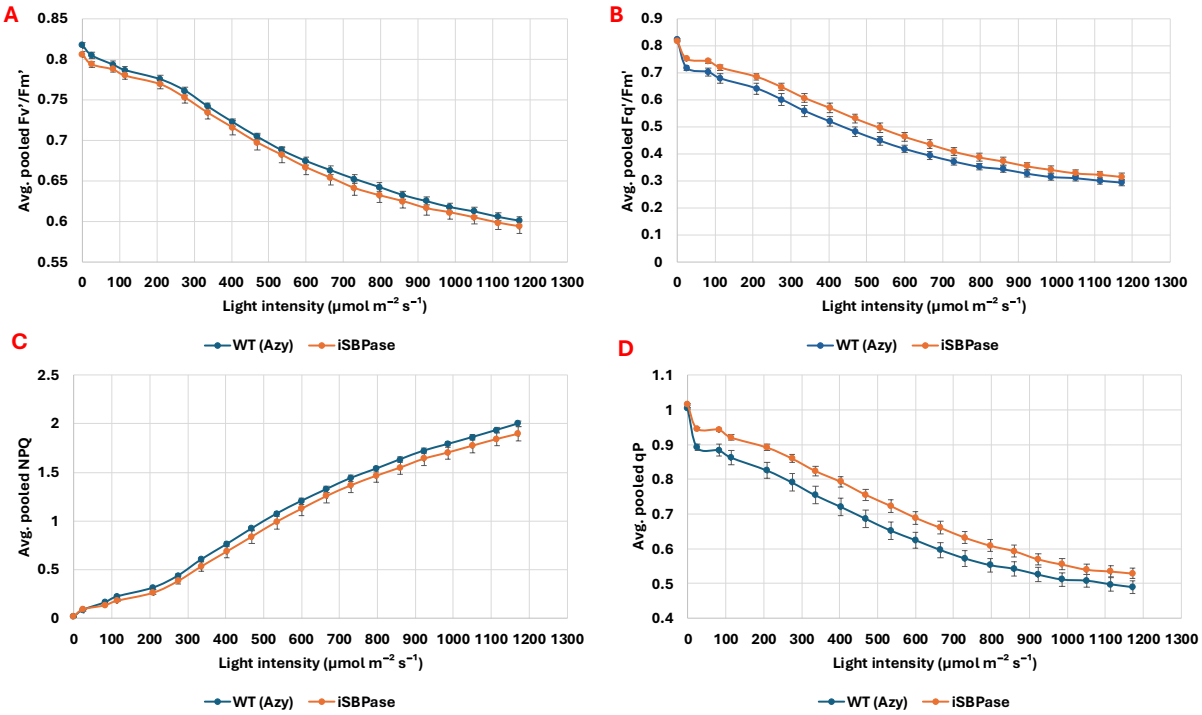

Figure 4.2. *Photosynthetic performance of iSBPase overexpression vs WT (Azy) fruit under increasing light intensities following 1-hour dark adaptation.* **(A)** *Fv'/Fm',* **(B)** *Fq'/Fm,* **(C)** *NPQ, and* **(D)** *qP. All parameters were measured across a light intensity gradient (0–1172 µmol m⁻² s⁻¹) in dark-adapted fruit. Each data point represents the mean calculated from pooled biological replicates (n ≥ 3). The error bars represent ± standard deviation. Blue lines indicate WT (Azy) controls; orange lines represent the iSBPase genotype*

As gas exchange measurements in fruit were not possible, chlorophyll fluorescence imaging was used to evaluate the effects of reducing SBPase activity on photosynthetic efficiency, PSII performance and energy absorption and dissipation dynamics. Two periods of dark adaptation were used: 1 hour and 24 hours. After a 1-hour dark adaptation, the Fv/Fm values were typical of non-stressed leaf material (above 0.8) for both genotypes (Fig. 4.3A).

The Fq'/Fm' data (Fig. 4.3B) showed there were statistical differences ($p < 0.05$) between the genotypes from 25 to 114 µmol m^{-2} s^{-1}. These results show that the iSBPase genotype has an effect on improving photosynthetic efficiency. 'Near significant' differences ($p < 0.07$) were observed after 114 µmol m^{-2} s^{-1} through to the intermediate light intensities (approximately 400 µmol m^{-2} s^{-1}).

The iSBPase genotype showed reduced NPQ values compared to the wild-type azygous controls (Fig. 4.3C); however, statistical analysis conducted indicated there were only significant differences between genotypes between 0, 82 and 114 µmol m^{-2} s^{-1}, with the

iSBPase genotype outperforming the control at these intensities ($p < 0.05$). The qP values declined with light intensity in both genotypes, with the wild-type genotypes having a steeper decline (Fig. 4.3D). The iSBPase genotype consistently showed higher qP values than the wild-type plants, particularly at moderate to high irradiance, which would point to an increased proportion of open PSII centres in transgenic tissue. Moreover, statistical tests conducted on the data at specific light intensities showed statistical significance between the genotypes from 0 $\mu mol^{-2}\ s^{-1}$ to 730 $\mu mol^{-2}\ s^{-1}$ ($p < 0.05$).

4.3.1.3 24-Hour Dark Adaptation of The Fruit

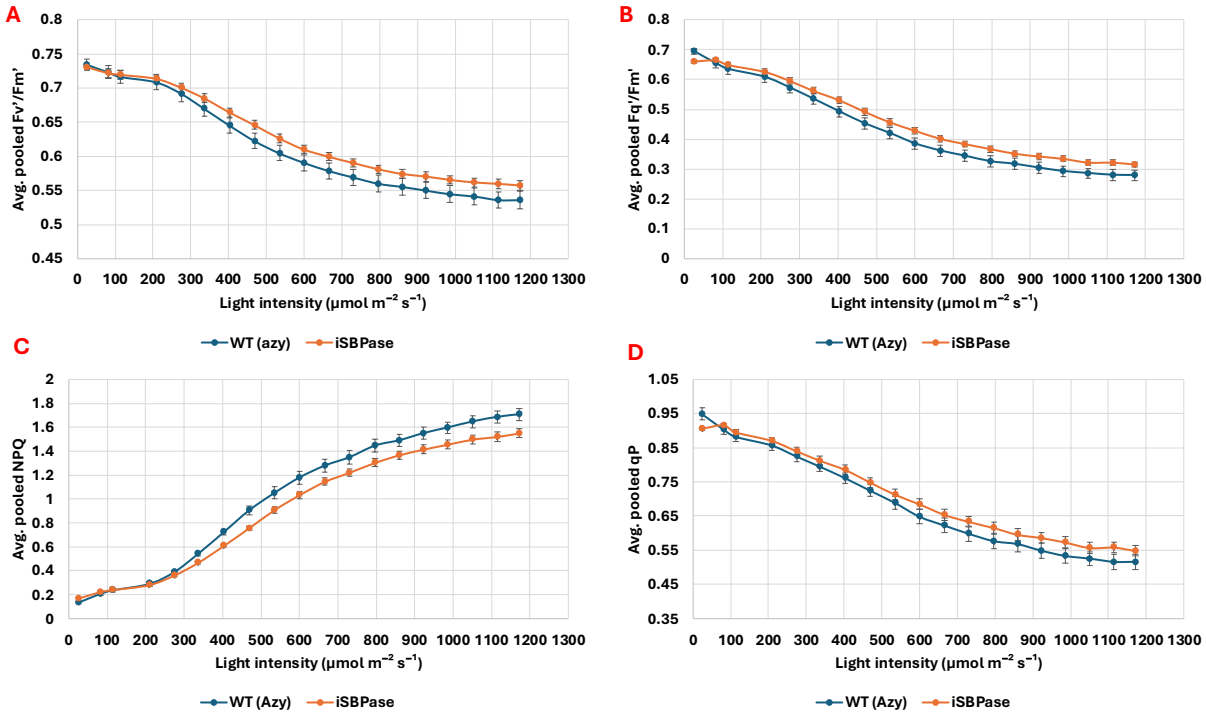

Figure 4.3. *Photosynthetic performance of WT (Azy) and iSBPase genotypes under varying light intensities following 24-hour dark adaptation of excised fruit. Chlorophyll fluorescence parameters were measured across increasing actinic light intensities (0–1172 µmol m⁻² s⁻¹).* (**A**) *Fv'/Fm',* (**B**) *Fq'/Fm',* (**C**) *NPQ, and* (**D**) *qP. Data is plotted as the average of pooled biological replicates (n ≥ 3) with error bars representing SD. The first data point (at 0 µmol m⁻² s⁻¹) for both genotypes was removed from all figures due to an error while recording. Blue lines indicate the WT (Azy) genotype; orange lines represent the iSBPase genotype.*

After a 24-hour dark adaptation, the Fv'/Fm' results showed no statistically significant differences throughout (Fig. 4.4B). The Fq'/Fm' values also decreased at a similar rate in response to the increasing light intensity in both WT (Azy) and iSBPase lines (Fig. 4.4B); however, some significant and near-significant differences were observed throughout the intermediate to high-light intensities (403 to 1115 $\mu mol\ m^{-2}\ s^{-1}$) ($p < 0.1$).

The NPQ results (Fig. 4.4C) showed that the iSBPase fruit had lower numerical NPQ values across most light intensities; these differences were shown to be statistically significant between intensities 275 to 1172 µmol m^{-2} s^{-1}. The qP data (Fig. 4.4D) showed that there was a statistical difference between the genotypes at 25 µmol m^{-2} s^{-1}. However, no statistically significant differences were observed at any other light intensity. An error occurred at the onset of the experiment, which resulted in anomalous data at 0 µmol m^{-2} s^{-1}; thus, the data point was removed.

4.3.1.4 Fruit Photosynthetic Response to Chilling

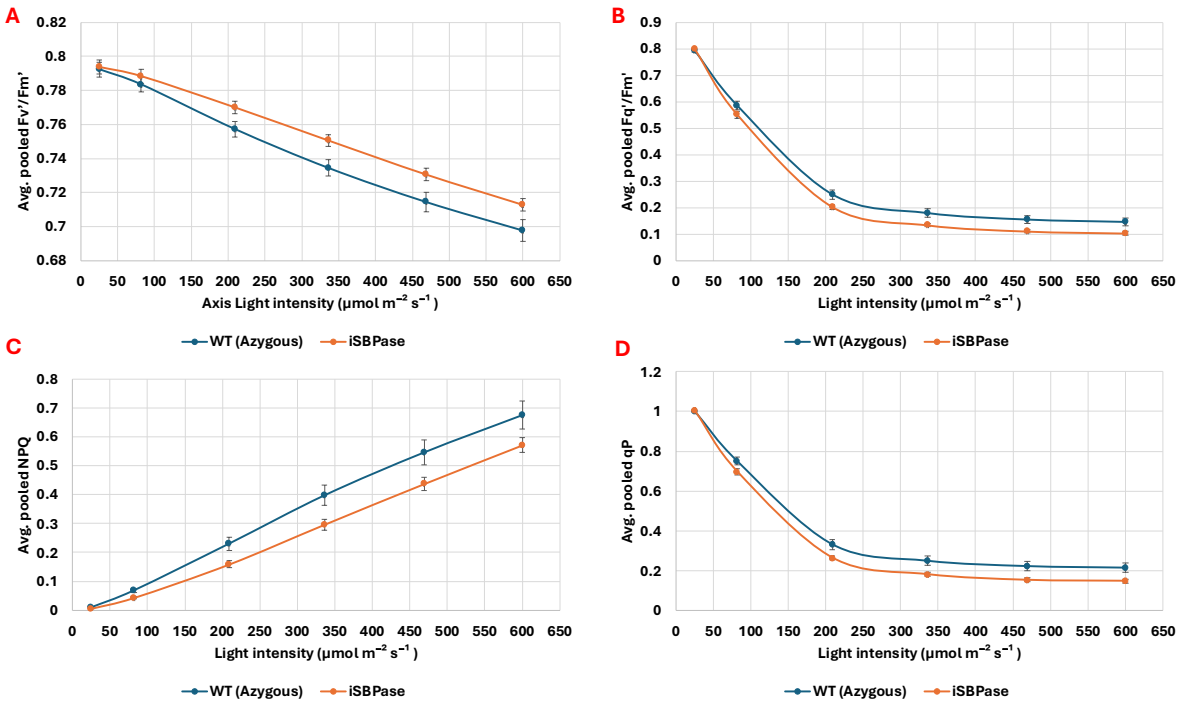

Figure 4.4. *Chlorophyll fluorescence parameters in wild-type azygous and iSBPase (SBPase RNAi knockdown) fruit lines following 12-hour dark adaptation at 5°C to ascertain the fruit's response to a chilling treatment. Four chlorophyll fluorescence parameters were measured:* **(A)** *Fv'/Fm',* **(B)** *Fq'/Fm',* **(C)** *NPQ and* **(D)** *qP. Data is shown as mean ± SD from pooled biological replicates (n ≥ 3). Statistically significant differences were observed between the genotypes above 209 µmol m^{-2} s^{-1} within all measured parameters. Blue lines indicate the WT (Azy) genotype; orange lines represent the iSBPase genotype.*

Photosynthesis is highly temperature-sensitive, and the CBB cycle can be a key limitation at low temperatures. Therefore, to assess the impact of chilling stress on fruit with reduced SBPase expression, chlorophyll fluorescence parameters were measured in wild-type and

iSBPase lines following a 12-hour period in the dark at 5°C. Plants were then exposed to increasing actinic light intensities, and the fluorescence responses were recorded.

The Fv'/Fm' mean values (Fig. 4.5A) past 209 µmol m^{-2} s^{-1} all showed statistical significance when compared to the control. The NPQ data (Fig. 4.5C) showed a similar trend where the iSBPase lines outperformed the wild-type, although this trend is observable at every light intensity. The Fq'/Fm' (Fig. 4.5B) and the qP data (Fig. 4.5D) together followed a similar trend where the control genotype performed better than the iSBPase genotype at and beyond a light intensity of 209 µmol m^{-2} s^{-1}.

4.3.2 Carotenoid Content and Sugar Content by Brix Assay

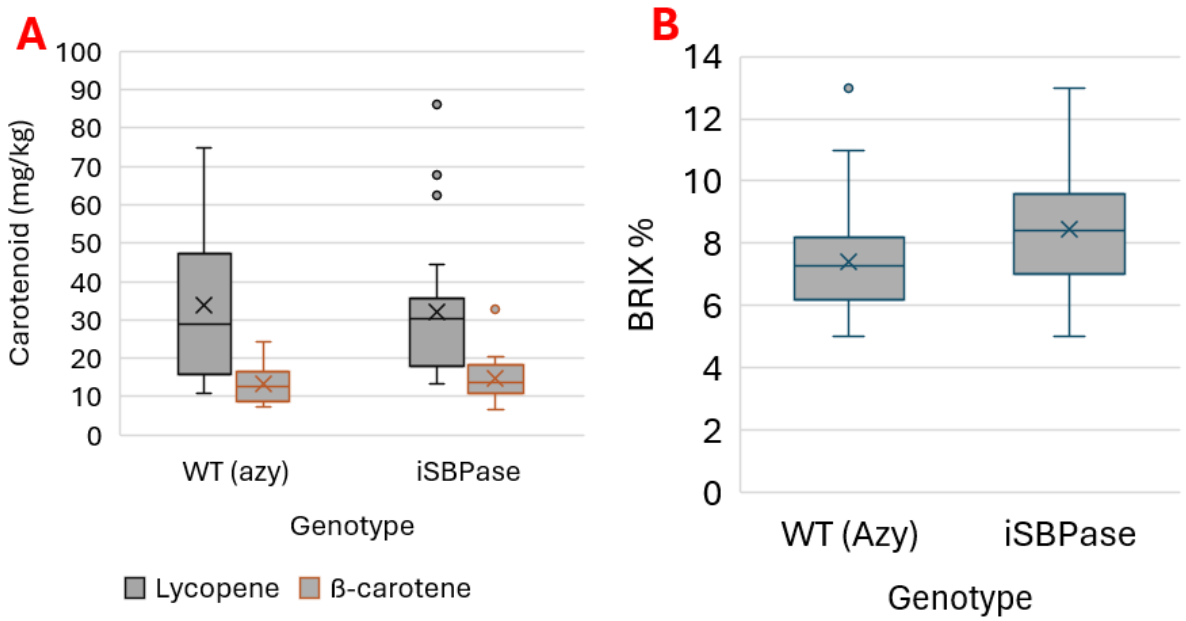

Figure 4.5. (A) *Carotenoid content in WT (azy) and iSBPase tomato genotypes measured via extraction assay. Boxplots show concentrations (mg/kg fresh weight) of lycopene (grey) (p = 0.79) and β-carotene (orange) (p = 0.80) in wild-type azygous and transgenic iSBPase fruit collected 7 days post-ripening. The central line represents the median, boxes indicate the interquartile range, whiskers show the range excluding outliers, and crosses (×) denote the mean. Outliers are represented as individual points.* **(B)** *A box and Whisker plot showing the Sugar Content by Brix Analysis data from both genotypes, transgenic and wild-type azygous (p = 0.00004).*

Carotenoid profiling of ripe fruit revealed no statistically significant differences in either lycopene or β-carotene concentrations between the WT (Azy) and iSBPase genotypes (Fig. 4.6A). Lycopene levels in WT (Azy) fruit ranged widely, with a median concentration of 28.78 mg/kg and a broader interquartile range than iSBPase fruit, which had a slightly lower median

of 30.26 mg/kg. However, a comparison between the WT (Azy) and iSBPase samples resulted in no significant difference in lycopene concentration between the groups ($p = 0.79$).

Similarly, fruit β-carotene concentrations were not significantly affected by SBPase suppression. WT (Azy) fruit had a median β-carotene content of 12.59 mg/kg, while iSBPase fruit showed a median of 13.12 mg/kg. This difference did not reach statistical significance ($p = 0.80$). Thus, lycopene and β-carotene levels remained within the expected biological range for red-ripe fruit.

Additionally, to assess the impact on sugar accumulation when significantly reducing SBPase expression in fruit, brix measurements were performed on juice extracted from red-ripe fruit from iSBPase RNAi lines and their corresponding wild-type controls (Fig. 4.6B). °Bx, an estimate of soluble sugar content, was significantly altered in the transgenic lines compared to WT (Azy).

The data revealed that the iSBPase knockdown fruits exhibited a clear and statistically significant increase in Brix values relative to the WT (Azy) controls. The median Brix in iSBPase fruit was elevated, and the interquartile range (IQR) shifted upward, indicating a general increase in sugar content across biological replicates. Notably, the mean Brix value obtained from the iSBPase fruit was approximately 8.46%, compared to 7.4% in the WT (Azy). The distribution in the iSBPase group also demonstrated an extended upper whisker and higher maximum values, reaching up to 13%, suggesting enhanced sugar accumulation in a subset of the fruit.

Statistical analysis using a two-tailed Student's t-test confirmed that the observed difference was highly significant ($p = 0.00004$), supporting the genotypic effect of SBPase suppression on fruit sugar content.

4.3.3 Fruit Biomass Measurements

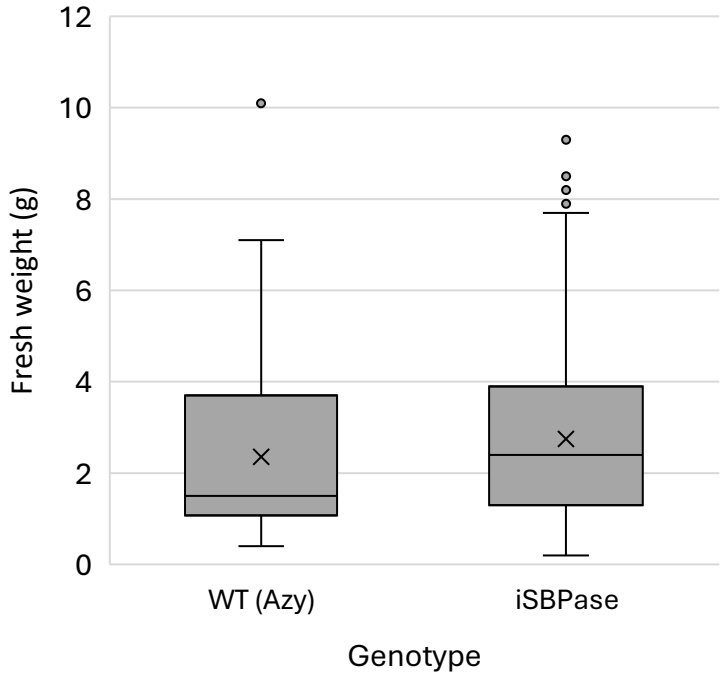

Figure 4.6. *The average fresh ripe fruit biomass was pooled by genotype and then averaged. Error bars represent standard deviation. (p = 0.148).*

To determine whether suppression of SBPase impacts fruit development, I measured the fresh weight of fully ripe fruit from the wild-type genotype and the transgenic genotype (iSBPase). Ripe fruits were harvested at maturity under identical growth room conditions and weighed individually to obtain fresh weight measurements.

Analysis of fresh fruit weight revealed no statistically significant difference between WT (Azy) and iSBPase fruit (Fig. 4.7). The median fresh weight of WT (Azy) fruit was 1.5 g, while iSBPase fruit were slightly heavier at 2.4 g. The interquartile ranges were comparable between genotypes, although iSBPase fruit exhibited a broader distribution, including more extreme values and a higher frequency of upper-range outliers. Despite this variation, the means (WT (Azy): 2.35 g; iSBPase: 2.75 g) were not significantly different when assessed by an unpaired two-tailed *t*-test ($p > 0.05$), indicating that silencing SBPase does not adversely affect final fruit mass.

These findings suggest that, under the tested environmental and developmental conditions, the reduction of SBPase expression in the iSBPase lines does not significantly impair fruit biomass accumulation. This outcome supports the hypothesis that SBPase activity may not be rate-

limiting for carbon allocation or that compensatory metabolic adjustments may mitigate the impact of reduced SBPase function.

4.4 Discussion

Leaf fluorescence light response curves confirmed that the targeted fruit-specific knockdown did not impair vegetative photosynthesis. In fruit, my data revealed increases in photosynthetic performance in iSBPase lines at room temperature, which was generally assessed through PSII efficiency. Additionally, a reduction in PSII efficiency was observed after a chilling treatment. NPQ was consistently lower in iSBPase plants across temperatures, suggesting a disconnect between photoprotective regulation and photochemical processes. Reduced photosynthetic performance was generally anticipated based on previous leaf studies where diminished SBPase activity restricted RuBP regeneration and impaired carbon assimilation. Despite these photochemical and non-photochemical changes, carotenoid levels and fruit biomass remained unaffected. By contrast, the sugar content was significantly increased, suggesting that targeted reduction of SBPase in fruit tissues can enhance sugar accumulation without compromising growth.

4.4.1 iSBPase Increases PSII Efficiency at Ambient Temperatures but Not Under Chilling

The analysis of chlorophyll fluorescence suggests that the targeted reduction of SBPase in fruit did not significantly affect the photochemistry of PSII in leaves. When various parameters were assessed (Fv′/Fm′, Fq’/Fm’/ΦPSII, NPQ, and qP), the performance of iSBPase leaves was similar to that of wild-type azygous controls. This indicates that photosynthetic efficiency in leaves remained unaffected by the SBPase reduction in fruit. The lack of influence on PSII photochemistry in leaves further suggests that the RNAi aspect of the construct's design, which specifically targeted SBPase suppression in the fruit tissue, was successful. Overall, these results indicate that the genetically induced knockdown of SBPase in tomato fruit does not impact leaf PSII photochemistry at this stage of development. This observation highlights the usefulness of tissue-specific promoters in studying the roles of photosynthetic enzymes within complex sink-source interactions and the resilience of leaf photochemistry when there are localised disturbances in fruit metabolism. It must be noted that it remains possible that more

significant effects could arise under conditions where the demand and supply of carbon are closely interconnected, such as during prolonged high light exposure, nutrient scarcity, or at later developmental stages when fruit act as dominant sinks. Exploring these scenarios in future experiments could continue to provide a comprehensive understanding of the larger impacts of alterations in fruit photosynthetic capacity on distal plant organs.

iSBPase should have a reduced regeneration capacity for RuBP, as leaf studies have shown the occurrence of lower CO_2 assimilation, especially under higher light (Lefebvre *et al.*, 2005; Ding *et al.*, 2016, 2018; Driever *et al.*, 2017). It is anticipated that this will be detectable as a reduced Fq'/Fm', more reduced PSII, and this may be associated with high NPQ.

The results for both 1-hr and 24-hr dark adaptation were generally consistent with each other and demonstrated that while SBPase is crucial for RuBP regeneration in leaf CBB cycle, its knockdown in fruit did not lead to a uniform suppression of photosynthetic light reactions. Instead, a nuanced pattern emerged, revealing subtle but biologically significant changes in PSII efficiency, photoprotection, and reaction centre openness across varying light conditions.

The Fv/Fm value was typical of healthy, non-stressed plant tissue (>0.8) (Bartold and Kluczek, 2024). This suggests that the maximum PSII efficiency under light-adapted conditions was largely preserved in the knockdown lines. This primarily indicates that knocking down SBPase does not cause the fruit any metabolic stress at ambient temperatures, unlike in leaves (Ding *et al.*, 2018).

The Fq'/Fm indicated that effective PSII quantum yield was temporarily enhanced in the knockdown lines under sub-saturating light. Such a response might reflect altered stromal metabolism, which reduces the demand for ATP and NADPH, maintaining a more oxidised plastoquinone pool and sustaining higher quantum yields when wild-type controls begin to exhibit efficiency losses. However, this advantage dissipated at higher light intensities, where both genotypes converged, suggesting that SBPase becomes a significant bottleneck only when electron transport rates near their capacity, consistent with previous work. The NPQ response further supports the idea that iSBPase fruit go through a rebalancing rather than a complete impairment of energy dissipation. As there were lower NPQ values in the iSBPase genotype, this may indicate slightly reduced activation of photoprotective mechanisms during the early light response, potentially due to a less reduced electron transport chain. Alternatively, this could reflect altered proton motive force dynamics linked to stromal metabolic constraints. The

lack of significant differences at higher irradiances, however, suggests that the chloroplasts in the fruit are capable of compensating effectively, thus preserving overall photoprotection under strong light stress.

The most striking finding was the behaviour of qP, which decreased with increasing irradiance in both genotypes but at a much slower rate in iSBPase fruit. The knockdown genotype qP values demonstrated that a greater proportion of PSII reaction centres remained open across most of the light gradient. This could indicate a redox state in which QA is more oxidised, possibly due to reduced CBB cycle flux lowering downstream sinks for NADPH, which redirects electron flow toward alternative pathways such as cyclic electron flow or oxygen reduction. This is less consistent with the expectation of a greater reduction of PSII.

The dissociation between elevated qP, unchanged Fv'/Fm', and largely unchanged NPQ after a 1-hour dark adaptation is noteworthy. This pattern indicates that the greater openness of PSII reaction centres does not translate into enhanced photochemical efficiency or altered capacity for non-photochemical energy dissipation. Instead, the elevated qP likely reflects a shift in the QA redox state driven by broader metabolic adjustments influencing electron flow, rather than a genuine increase in photochemical performance.

These results indicate that reducing SBPase activity in fruit does not lead to the expected decline in overall photosynthetic performance. Instead, it enables adjustments in the regulation of electron transport and the balance of photochemical processes. The modest improvements observed in Fq'/Fm' and the consistent increases in qP at biologically relevant light intensities suggest a modified redox state of the photosynthetic system in iSBPase fruit. These findings emphasise that fruit chloroplasts function under different metabolic constraints compared to leaves, integrating photosynthesis not only with carbon fixation but also with biosynthetic activities. Future research combining gas exchange measurements, stromal metabolite profiling, and PSI electron transport assessments will be necessary to determine whether the observed changes reflect genuine increases in CO_2 assimilation capacity or represent altered energy allocation strategies specific to fruit photosynthesis.

The differences observed following the 24-hour dark adaptation show responses that were not seen during the shorter 1-hour treatment. This indicates that the duration of dark adaptation significantly influences the balance between photoprotective mechanisms and photochemical regulation in the chloroplasts of fruit under the conditions tested.

The variation in qP behaviour is particularly interesting, as with just 1 hour of dark adaptation, iSBPase fruit showed considerable differences in qP across multiple light intensities, suggesting a less efficient reopening of PSII reaction centres during illumination. This may be due to insufficient relaxation of the NPQ processes and the persistence of metabolic redox conditions from the light phase. Under these circumstances, inconsistencies in stromal metabolism between genotypes would be amplified, as limitations in RuBP regeneration due to lower SBPase levels would directly reduce electron transport and reaction centre turnover. In contrast, after 24 hours of dark adaptation, the complete relaxation of both NPQ and stromal redox states likely reset PSII to a standardised baseline, diminishing the apparent differences in qP across lines. Essentially, prolonged dark adaptation might obscure the genotype-specific limitations on reaction centre dynamics by equalising the initial redox environment.

In fruit chloroplasts, which already face limited internal CO_2 availability, this reduction in NPQ could render PSII more susceptible to overexcitation, aligning with the slight but noticeable declines in Fq'/Fm'. Notably, since qP did not vary between genotypes after 24 hours, it suggests that the limitation in iSBPase fruit is not about keeping the reaction centre open but rather how the incoming excitation energy is allocated downstream once it reaches PSII.

Overall, these findings highlight that SBPase influences not only the flux of the CBB cycle but also the photoprotective dynamics of fruit photosynthesis. The observation that differences in qP are significant after a short recovery but diminish with extended dark adaptation suggests that fruit photosynthesis is particularly sensitive to SBPase limitations during rapid light changes, when incomplete metabolic relaxation amplifies differences in redox status between genotypes. However, over a longer duration, the regulatory issues are more evident in NPQ, underscoring the significance of energy dissipation capacity in maintaining fruit photochemistry under varying conditions. Importantly, while the 24-hour dark adaptation was effective in revealing subtle baseline differences in NPQ and Fq'/Fm', it does not mimic a natural scenario for fruit, which usually undergoes cycles of shading and light exposure. Rather, this extended dark phase served as an artificial way to reset photosynthetic regulation and elucidate the underlying genotype-specific variations.

The literature suggests that constitutive overexpression of SBPase improves chilling-induced oxidative stress in tomato plants (Ding, Wang and Zhang, 2017; Ding et al., 2016) and rice (Li et al., 2020). Additionally, the research conducted by Ding et al. (2016) showed that the constitutive silencing of SBPase via CRISPR-Cas9 results in plants with reduced tolerance to

chilling (Ding et al., 2016). SBPase is essential for the regeneration of RuBP in the CBB cycle and has been linked to enhanced carbon assimilation and ROS management under low-temperature stress. Here, I note that the outcome of lowered PSII efficiency (Fq'/Fm') in fruit is consistent with the original hypothesis. It is possible that chilling places SBPase in a position of greater control over CBB cycle turnover; its silencing causes a reduction in efficiency that was not observed at ambient temperatures.

One possible explanation for the discrepancy between my ambient temperature results and the literature involves the fundamental nature of fruit, as they are sink tissues with inherently lower photosynthetic activity than leaves, which may not rely as heavily on SBPase-mediated CBB cycle function. In fruit, metabolic compensation or alternate photoprotective pathways (e.g., increased reliance on the xanthophyll cycle, cyclic electron flow, or antioxidant systems) may greatly reduce the impact of SBPase suppression.

In summary, the reduction of SBPase abundance in tomato does not impact leaf photochemistry. This suggests the construct's fruit specificity and the resilience of photosynthesis within source organs when there are modifications to photosynthesis within sink organs. Under ambient conditions, fruit photochemistry remained largely intact, with only nuanced adjustments in PSII regulation. This was evident by the presence of elevated qP and reduced NPQ values in both ambient experiments. These results suggest a shift in redox and electron transport balance rather than the expected outright loss of efficiency seen in other studies (Lawson *et al.*, 2006; Ding *et al.*, 2016, 2018).

On the other hand, under chilling stress, SBPase silencing was associated with a measurable decline in PSII efficiency (Fq'/Fm'). This result is consistent with the enzyme's established role in RuBP regeneration and mitigation of oxidative stress (Lawson *et al.*, 2006; Ding *et al.*, 2016; Ding, Wang and Zhang, 2017). This divergence brings attention to how the unique metabolic context of fruit plastids can compensate for a significant reduction in SBPase activity at ambient temperatures but can worsen PSII efficiency under stress conditions. Overall, these findings strongly suggest that SBPase acts as a conditional and optional control point in fruit photosynthesis. It is optional under ambient conditions but becomes critical under chilling stress. Thus, these results show the importance of context when interpreting the functional roles of CBB cycle enzymes in non-foliar tissues. When taking into account my study involving the overexpression of SBPase, both results show that differentially expressing this enzyme results

in near identical Fv'/Fm' and NPQ results. This highlights its potential to provide fruit-specific metabolic adjustments for stress resilience.

4.4.2 iSBPase Increases Sugar Concentration Without Impacting Biomass

My results imply that the biosynthesis of carotenoids during the ripening process is largely resilient to moderate reductions in CBB cycle activity. Although triose phosphates from the CBB cycle are involved in feeding the Methylerythritol phosphate (MEP) pathway, which supplies precursors for carotenoid production (Lichtenthaler, 1999; Rodríguez-Concepción & Boronat, 2002), the lack of observable changes indicates that this connection is not a significant limiting factor under the tested conditions. This insensitivity aligns with the developmental reprogramming of plastids that occurs during ripening; specifically, chloroplasts transition into chromoplasts, and photosynthetic activity declines rapidly (Laval-Martin et al., 1975; Lado et al., 2016). At this stage, pigment synthesis is primarily regulated by ethylene signalling and the transcriptional control of pathway enzymes like PSY1, PDS, and ZDS (Fraser et al., 1994; Alba et al., 2000; McQuinn et al., 2020), which operate largely independently of ongoing carbon assimilation.

The lack of impact on carotenoid levels is noteworthy when compared to the significant increase in Brix observed in the same iSBPase lines, indicating that sugar metabolism is more responsive to changes in carbon flux than pigment production. This difference likely reflects distinct regulatory mechanisms at work: while soluble sugars dynamically adjust to fluctuations in source-sink relationships and carbon allocation (Carrari et al., 2006), carotenoid biosynthesis is tightly regulated by developmental cues. One possibility is that the suppression of SBPase altered carbon distribution within the fruit by shifting assimilates towards soluble carbohydrate accumulation, resulting in increased sugar concentrations without substantially affecting pigment levels.

Additionally, the photosynthetic fluorescence data support this interpretation. The iSBPase fruit showed a significant rise in qP at moderate light levels after dark adaptation, along with trends towards higher Fq'/Fm' and lower NPQ under similar conditions. These observations suggest a slight improvement in the efficiency of light utilisation, particularly at light intensities

similar to the growth environment (~400 μmol m^{-2} s^{-1}). Though these changes may seem small on an individual measurement basis, they could accumulate over developmental time, creating a consistent advantage for photosynthesis that leads to greater sugar accumulation. Moreover, the fact that these improvements were most pronounced after extended dark adaptation suggests that iSBPase fruit may be better prepared to take advantage of daily light exposure, potentially enhancing carbon assimilation throughout the photoperiod.

Crucially, these metabolic changes seem limited to the fruit itself. Chlorophyll fluorescence measurements from the leaves showed no significant differences between genotypes, indicating that the overall photosynthetic efficiency of the plant remained unchanged. This strongly suggests that the increased sugar content resulted from specific metabolic adjustments within the fruit rather than systemic changes in source activity. Altogether, the data support a model in which the suppression of SBPase disrupts carbon partitioning in the fruit, enhancing sugar accumulation while not affecting carotenoid production.

These findings highlight the different regulatory mechanisms governing sugar and carotenoid metabolism in ripening tomato fruit. While sugar levels are flexible and responsive to shifts in carbon allocation, carotenoid biosynthesis is robustly maintained by developmental programming. This separation has significant implications: moderate alterations to CBB cycle activity within the fruit may enhance sweetness without compromising colour or nutritional quality. Future investigations should explore carbohydrate partitioning, phloem transport, and sink metabolism in iSBPase lines to better understand the mechanisms behind this reallocation. Such studies could provide valuable insights into how adjustments in photosynthetic processes relate to fruit quality traits and the balance between developmental and metabolic controls.

My result suggests that fruit development is relatively resilient to upstream metabolic changes. Key developmental checkpoints or source-sink relationships may ensure fruit reaches a consistent final weight, even when internal metabolic pathways are altered. Another possibility is that any changes in carbon allocation throughout development are either balanced out over time or do not influence overall biomass to a degree that is detectable at harvest.

The broader spread in the iSBPase data, particularly the presence of more frequent upper outliers, may indicate some variability in how individual fruits responded to the suppression. This could indicate subtle shifts in carbon dynamics or developmental variability; however, the

absence of a consistent trend across the population suggests that the biological impact on fresh weight is likely minimal.

Interestingly, the increase in soluble sugar content, despite no change in fruit size, suggests a shift in how carbon is partitioned within the fruit. Rather than being used for further growth or structural expansion, more carbon may be retained as sugars. Alternatively, it could reflect reduced dilution due to subtle changes in water content or growth rate. In either case, the data show that fruit composition and fruit size are not always tightly linked, and in this context, SBPase suppression appears to enhance fruit sweetness without compromising yield.

4.5 Conclusion

The development of tomato lines with reduced SBPase expression has provided a unique opportunity to assess the impact of CBB cycle capacity on fruit photosynthesis and related traits. As anticipated, leaf fluorescence response curves showed no significant differences between the iSBPase lines and the control plants, indicating that the fruit-specific construct did not affect photosynthetic performance in the leaves. This confirms the tissue specificity of the promoter and assures that any observable phenotypic effects can primarily be attributed to the fruit's photosynthetic processes.

In the fruit, however, distinct genotype-dependent effects emerged. Following both 1-hour and 24-hour dark adaptations, the iSBPase lines demonstrated increased Fq'/Fm' and qP across a variety of light intensities, along with reduced NPQ induction. Additionally, chilling the fruits for 12 hours at 5 °C revealed genotype differences across all major fluorescence parameters, indicating that reduced SBPase might enhance photosynthetic efficiency under stressful conditions.

Biochemically, carotenoid content remained unchanged, highlighting that pigment accumulation does not significantly rely on SBPase activity. However, the average Brix was notably higher in iSBPase fruit, despite no significant changes in final biomass. This disconnect between assimilate production and growth underscores how a reduction in SBPase activity influences carbon metabolism within fruit without restricting yield.

In summary, this novel study shows that fruit exhibits less sensitivity to the downregulation of SBPase compared to leaves, maintaining considerable photochemical performance, and in some cases, even surpassing the control genotype. The significant increase in sugar accumulation suggests that fruit photosynthesis operates under different regulatory mechanisms than foliar photosynthesis. Additionally, this study suggests that fruit photosynthesis is primarily restricted by CO_2 availability rather than by the capacity of CBB cycle enzymes. The unexpected enhancement of sugar accumulation following SBPase knockdown challenges conventional expectations, revealing the complexity of metabolic regulation in non-foliar photosynthesis. This surprising result warrants further exploration to uncover the underlying mechanisms in addition to an assessment as to how it could be leveraged alongside other photosynthetic and metabolic modifications to improve crop quality.

From a practical standpoint, these findings possess clear agricultural implications. The increase in fruit sugar content without compromising biomass could be a powerful tool. As sweetness significantly influences consumer preferences and market value in tomatoes, strategies to boost endogenous sugar levels represent an appealing breeding and biotechnological opportunity. Moreover, given SBPase's central role in RuBP regeneration, similar strategies might apply to other fleshy fruit crops, such as peppers, cucumbers, and grapes, where photosynthetically active tissues also contribute to sugar accumulation. This novel research not only advances our fundamental understanding of fruit-specific photosynthesis but also highlights a promising pathway to improving crop quality and market competitiveness in crops of the future.

4.6 Bibliography

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5 Chapter 5: Overexpressing Fructose-1,6-bisphosphate Aldolase (FBPa) in Tomato Fruit to Determine Its Contribution to Photosynthetic Rate and Nutritional Quality

5.1 Introduction

A regulatory node within fruit-localised photosynthesis for potential improvement is Fructose-1,6-bisphosphate aldolase (FBPa). This enzyme plays an essential role in both the Calvin–Benson–Bassham (CBB) cycle and glycolysis. Within the CBB cycle, FBPA is responsible for catalysing the interconversion of fructose-1,6-bisphosphate (F1,6BP) into glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), in addition to contributing to RuBP regeneration. Regarding glycolysis, it provides substrates for sugar biosynthesis and other metabolic pathways (Raines, 2011; Lytovchenko et al., 2011). Studies in leaves suggest FBPA is a bottleneck and limits carbon flux through the CBB cycle. The overexpression of FBPA has been shown to reduce this carbon flux bottleneck and allow for an increase in net photosynthetic rate and biomass (Cai *et al.*, 2022), thus proving to be a gene of interest that can benefit industry and customers alike. However, FBPa's role and significance toward photosynthesis in green fruit tissue has not been determined.

The upregulation of FBPA in tomato fruit has the potential to increase the pool of triose phosphates available for sucrose and hexose biosynthesis. The increased production of glucose and fructose as a result of FBPA upregulation may influence fruit sweetness, osmotic potential, and sink strength, thus having a direct effect on fruit size and development (Yelle et al., 1991; Ho, 1996; Beauvoit et al., 2014). Increased availability of triose phosphates may also support secondary metabolism as both G3P and DHAP feed into the methylerythritol phosphate (MEP) pathway, the source of isoprenoid precursors for carotenoid biosynthesis (Fraser et al., 2007; Schauer et al., 2006). This raises the possibility that FBPA overexpression could enhance the accumulation of lycopene, an important nutritional and commercial trait. Furthermore, the

intermediates of glycolysis and the tricarboxylic acid cycle provide the substrates for flavonoids, ascorbate, and amino acids. This suggests that FBPA may influence fruit nutritional quality.

Additionally, by increasing the conversion of FBP into triose phosphates, FBPA overexpression could reduce the accumulation of sugar phosphates that otherwise impose inhibitory feedback on carbon metabolism (Stitt et al., 2010). In principle, this might stabilise carbon flow and minimise bottlenecks during fruit expansion and ripening. Thus, fruit-specific upregulation of FBPA may increase sink demand, altering the partitioning of photoassimilates from leaves to fruit, and influencing overall carbon economy. However, an unbalanced increase in FBPA activity may disturb the equilibrium of the CBB cycle and glycolysis, especially if not matched by coordinated adjustments in related pathways. Experimentation involving the differential expression of this enzyme in developing fruit is required to explore whether the same benefits can be achieved as observed in leaf studies.

In this chapter, fruit-specific transgenic tomato lines overexpressing FBPA were generated using the methods described in Chapter 2. Their photosynthetic and metabolic performance is assessed using chlorophyll fluorescence imaging, coupled with physiological and biochemical measurements of sugar accumulation, pigment content, and fruit development. The overall aim of this chapter is to determine whether fruit-specific FBPA overexpression enhances fruit-localised photosynthetic efficiency, and to assess whether increased carbon flux alters sugar accumulation, fruit weight, and carotenoid concentration.

5.2 Materials and Methods

The construct design and the growth conditions for the plant material can be found collated in Chapter 2.

The full material methods section can be found in Chapter 3 titled “Upregulating Sedoheptulose-1,7-bisphosphatase (SBPase) in Tomato Fruit to Increase Fruit Photosynthetic Rate”. For all results presented in this chapter, differences were considered statistically significant at the conventional threshold of $p < 0.05$.

5.3 Results

5.3.1 Chlorophyll Fluorescence Imaging

5.3.1.1 Leaf Tissue

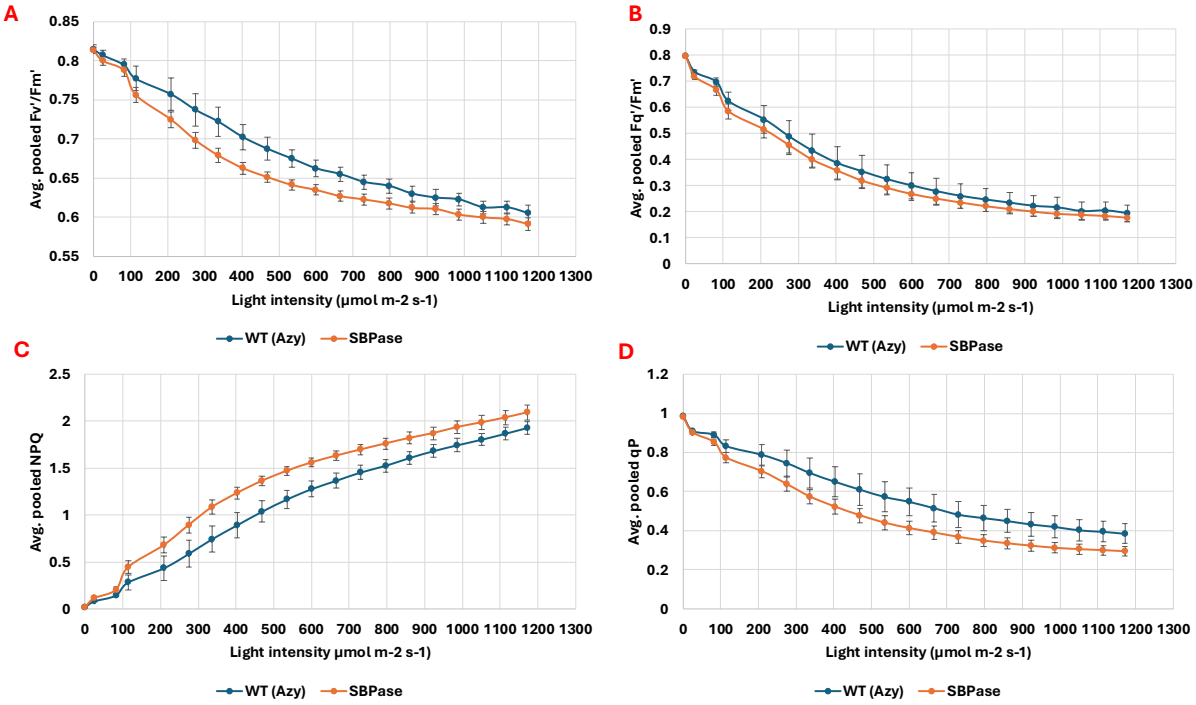

Figure 5.1. *A leaf fluorescence light response curve showing the* **(A)** *Maximum quantum yield of PSII photochemistry, Fv'/Fm',* **(B)** *Effective quantum yield of PSII, Fq'/Fm'/ΦPSII,* **(C)** *Non-photochemical quenching, NPQ, and* **(D)** *Photochemical quenching, qP. Results were obtained from the young and fully expanded leaves that were harvested and dark-adapted for 20 minutes before imaging. Each data point represents the mean calculated from pooled biological replicates (n ≥ 3). The error bars represent ± standard deviation. Blue lines indicate the WT (Azy) genotype; orange lines represent the FBPA genotype.*

The maximum quantum yield of PSII (Fv′/Fm′) (Fig. 5.2A) decreased progressively across light intensities in both genotypes, with FBPA leaves showing slightly lower values than WT (Azy) above 400 µmol m^{-2} s^{-1}, though not significantly. The effective quantum yield (Fq′/Fm') (Fig. 5.2B) was predominantly marginally higher in FBPA leaves, but again, not statistically significant. Leaf non-photochemical quenching (NPQ) (Fig. 5.2C) increased with light intensity, with FBPA showing a trend toward higher NPQ values after 300 µmol m^{-2} s^{-1}, though not statistically supported. Lastly, photochemical quenching (qP) (Fig. 5.2D) declined in both genotypes, with FBPA maintaining slightly higher values at most irradiances, also not significant.

Overall, my results showed that the observed differences between FBPA and WT (Azy) leaf tissue were small and not statistically significant.

5.3.1.2 1-Hour Dark Adaptation of The Fruit

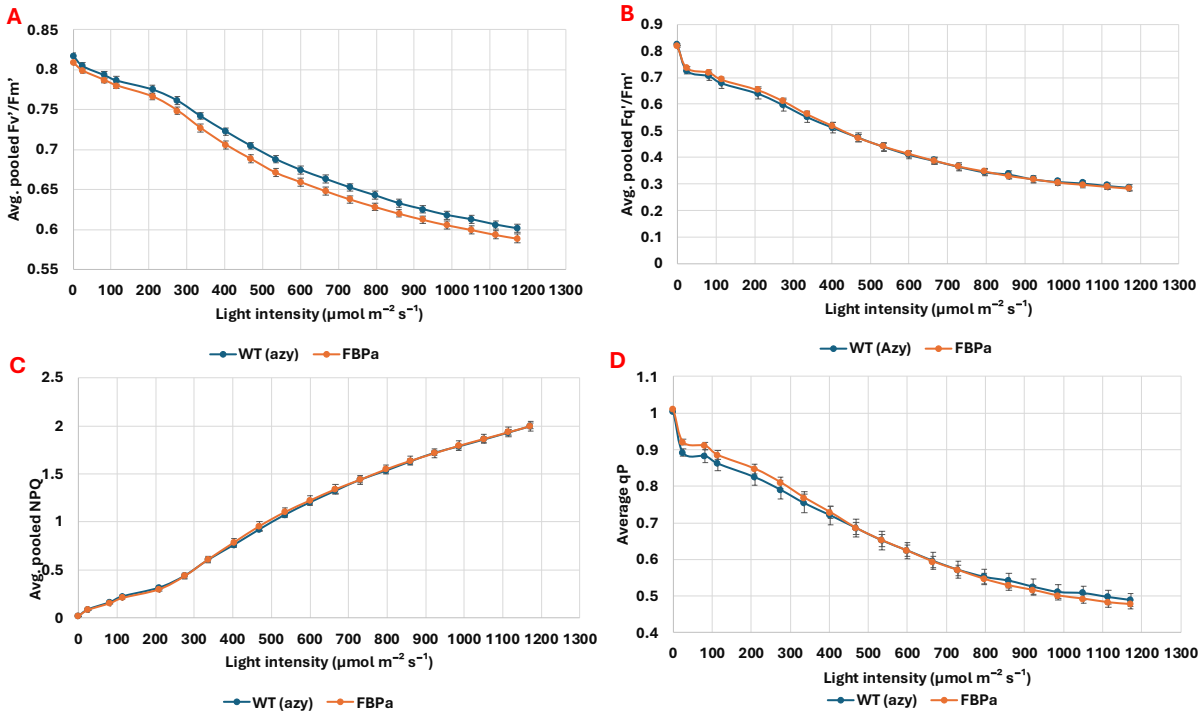

Figure 5.2. *A fluorescence light response curve showing FBPA and WT (azy) fruit under increasing light intensities following 1-hour dark adaptation.* **(A)** *Fv'/Fm',* **(B)** *Fq'/Fm',* **(C)** *NPQ, and* **(D)** *qP. All parameters were measured across a light intensity gradient (0–1172 µmol m⁻² s⁻¹). Data is shown as mean ± SD from pooled biological replicates (n ≥ 3). Blue lines indicate the WT (Azy) genotype; orange lines represent the FBPA genotype.*

I measured four chlorophyll fluorescence parameters in both FBPA overexpressing fruit and WT (Azy) controls across a range of light intensities (0–1172 µmol m^{-2} s^{-1}) following a 1-hour dark adaptation period. In dark-adapted conditions (0 µmol m^{-2} s^{-1}, both FBPA overexpressing and WT azygous fruit displayed high initial Fv/Fm values (~0.8–0.83) typical for most terrestrial plant species (Fig. 5.3A) (Bartold and Kluczek, 2024). Although the FBPA genotype consistently exhibited slightly lower Fv'/Fm' values compared to the WT genotype throughout, these differences were not statistically significant (Fig. 5.3A). However, at and between 336-797 µmol m^{-2} s^{-1}, the differences showed significance to the $p < 0.1$ level, where WT (Azy) outperformed the FBPA genotype.

Overall, across all measured chlorophyll fluorescence parameters (Fig. 5.3), FBPA overexpressing fruit displayed trends nearly indistinguishable from those of wild-type azygous controls across the full range of light intensities tested at the $p < 0.05$ level.

5.3.1.3 24-Hour Dark Adaptation of The Fruit

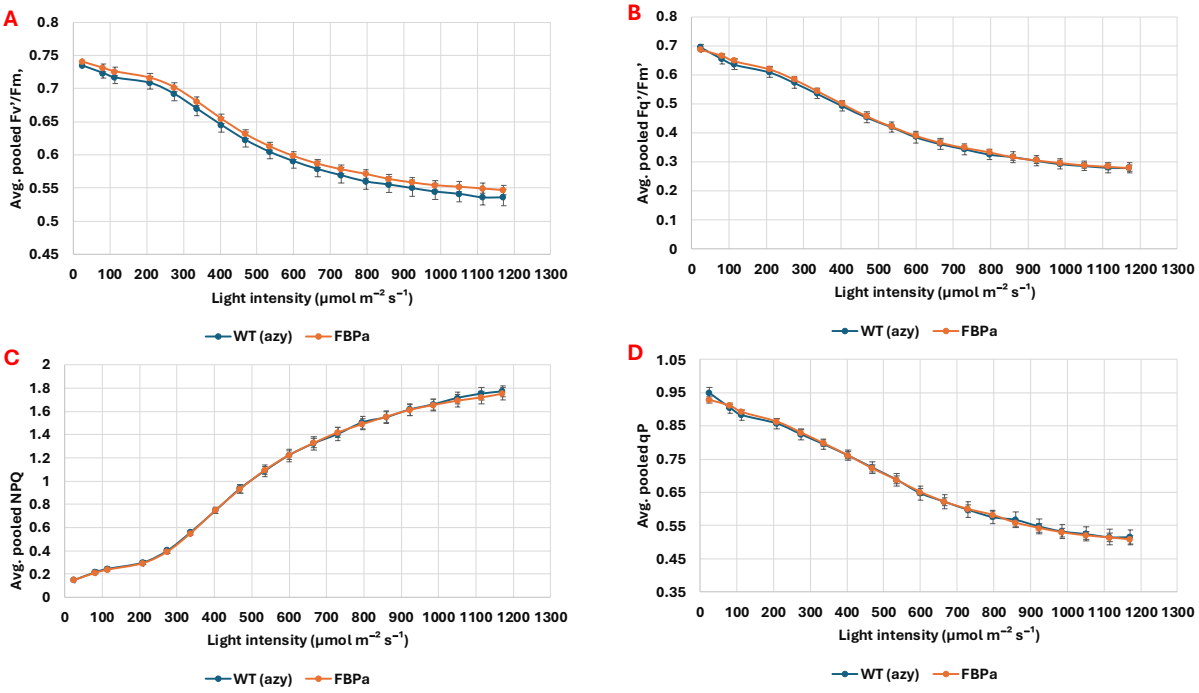

Figure 5.3. *Chlorophyll fluorescence parameters were measured across increasing light intensities (0–1172 µmol m^{-2} s^{-1}) following 24-hour dark adaptation of excised fruit.* (**A**) *Fv'/Fm',* (**B**) *Fq'/Fm',* (**C**) *NPQ, and* (**D**) *qP. Data is plotted as the average of pooled biological replicates ($n \geq 3$) with error bars representing SD. No statistically significant differences were observed between WT (Azy) and FBPA plants at any light intensity. The first data point (0 µmol m^{-2} s^{-1}) for both genotypes was removed from all figures due to an error while recording. Blue lines indicate the WT (Azy) genotype; orange lines represent the FBPA genotype.*

Following a 24-hour dark adaptation period, the FBPA fruit consistently had a slightly higher mean Fv'/Fm' values than WT (Azy) over much of the light range (Fig. 5.4A); however, these differences were within the range of biological variability and not statistically significant. Regarding Fq′/Fm′, the extent of the differences between genotypes was minor and not significant (Fig. 5.4B). This result is also true for the NPQ (Fig. 5.4C) and qP results (Fig. 5.4D).

Overall, FBPA fruit displayed photosynthetic light response profiles closely resembling those of the WT (Azy) fruit under these experimental conditions. While small, consistent shifts in Fv'/Fm' mean values suggested a trend toward marginally higher Maximum PSII efficiency in FBPA overexpressing fruit, these differences were not supported by statistical analysis.

5.3.1.4 Fruit Photosynthetic Response to Chilling Treatment

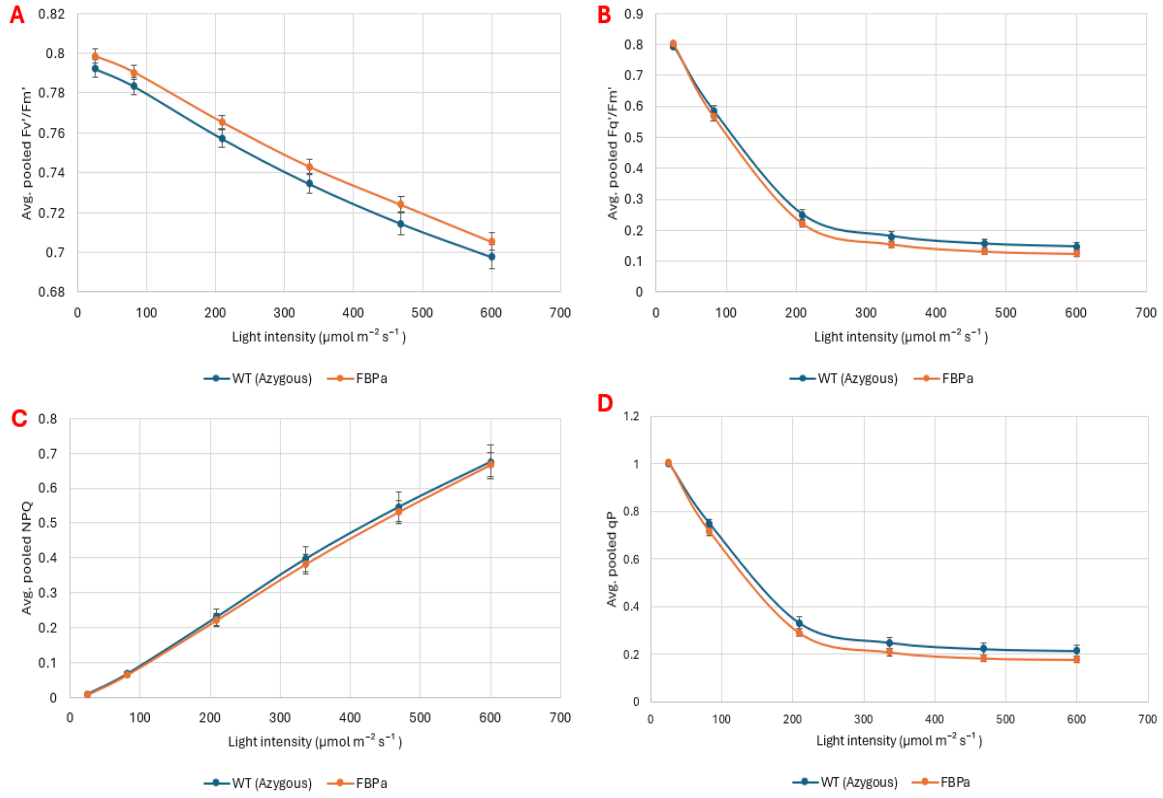

Figure 5.4. *Chlorophyll fluorescence parameters in wild-type (Azygous) and FBPA fruit lines following 12-hour dark adaptation at 5°C to ascertain the fruit's response to a chilling treatment. Four chlorophyll fluorescence parameters were measured:* (**A**) *Fv'/Fm',* (**B**) *Fq'/Fm',* (**C**) *NPQ, and* (**D**) *qP. Data is shown as mean ± SD from pooled biological replicates (n ≥ 3). No statistically significant differences were observed across all light intensities. Blue lines indicate the WT (Azy) genotype; orange lines represent the FBPA genotype.*

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After the fruit were chilled at 5°C for 12 hours, chlorophyll fluorescence measurements of FBPa-overexpression fruit and azygous controls across increasing light intensities showed no statistically significant differences for any parameter assessed across all intensities (Fig. 5.5).

5.3.2 Carotenoid Content and Sugar Content by Brix Analysis

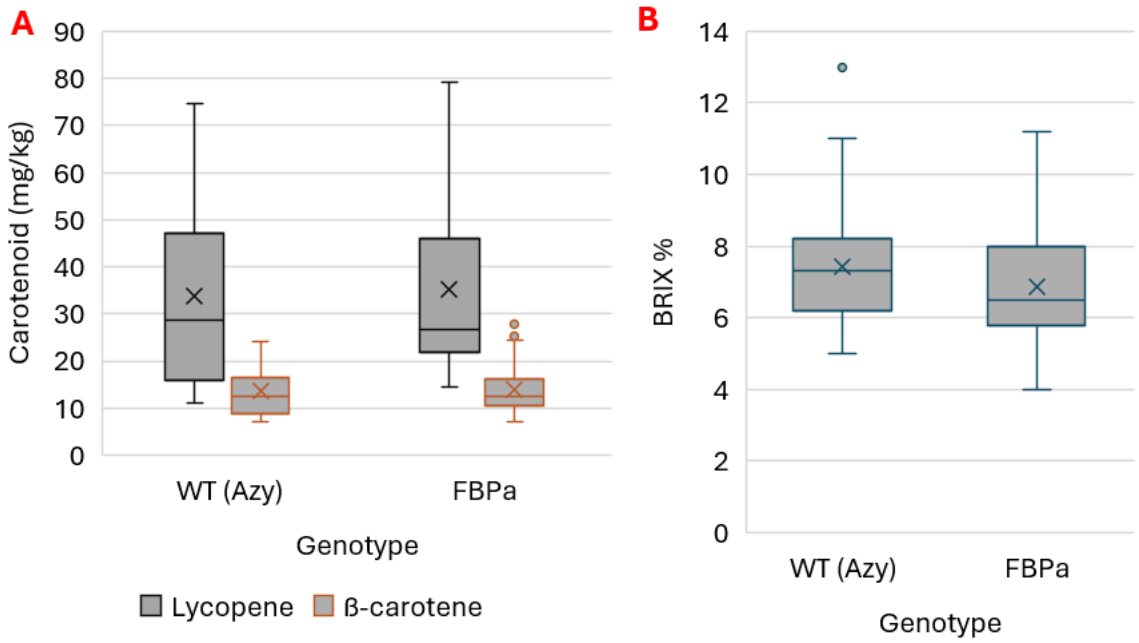

Figure 5.5. (A) *Carotenoid content in WT (Azy) and FBPA tomato genotypes measured via extraction assay. Boxplots show concentrations (mg/kg fresh weight) of lycopene (grey) and β-carotene (orange) in wild-type azygous and transgenic FBPA fruit collected 7 days post-ripening (p = 0.8 for both). The central line represents the median, boxes indicate the interquartile range, whiskers show the range excluding outliers, and crosses (×) denote the mean. Outliers are represented as individual points.* **(B)** *A box and Whisker plot showing the Sugar Content by Brix Analysis data from both genotypes, transgenic and wild-type azygous (p = 0.0106).*

Carotenoid (lycopene and β-carotene) content and the concentration of total dissolved solids measured by specific gravity (°Bx), which primarily indicates soluble sugar concentration, were assessed in ripe fruit from wild-type azygous (WT (Azy)) and FBPa-overexpressing tomato plants. The quantification of carotenoids showed no statistically significant differences between the genotypes for either lycopene or β-carotene (p = 0.8 for both) (Fig. 5.6A). In WT (Azy) fruit, lycopene was the predominant carotenoid, with a mean concentration of 33.7 mg/kg, a median of 28.8 mg/kg, and a wide distribution ranging from approximately 11.0 mg/kg to 74.7 mg/kg. Similarly, FBPa-overexpressing lines displayed a lycopene mean of 35.1 mg/kg, median of 26.8 mg/kg, and a comparable distribution (minimum: 21.9 mg/kg, maximum: 79.2 mg/kg).

β-carotene levels were also comparable between genotypes. WT (Azy) fruit showed a mean of 13.5 mg/kg and a median of 13.5 mg/kg, while FBPA lines had an almost identical mean of 13.9 mg/kg and a median of 13.9 mg/kg. These findings suggest that FBPA overexpression

does not significantly impact carotenoid biosynthesis or accumulation under the tested conditions.

However, fruit sugar concentration, measured as Brix, was significantly lower in FBPa-overexpressing geneotype compared to WT (Azy) controls (Fig. 5.6B). WT (Azy) fruits had a mean Brix of 7.4% and a median of 7.3%, with values ranging from 5.0% to 11.0%, and one notable outlier at 13.0%. Conversely, FBPA fruits exhibited a mean Brix of 6.88% and a median of 6.5%, with a broader range extending from 4.0% to 11.2%.

5.3.3 Fruit Biomass Measurements

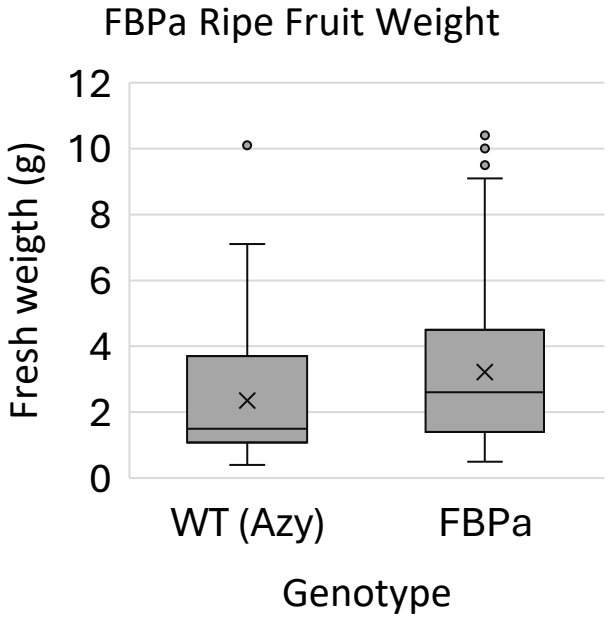

Figure 5.6. *The average weight of fresh, ripe fruit was pooled by genotype and then averaged. The central line represents the median, boxes indicate the interquartile range, whiskers show the range excluding outliers, and crosses (×) denote the mean. Outliers are represented as individual points (p = 0.0089).*

The fresh weight of ripe fruit from WT (Azy) and FBPA transgenic lines was measured. As shown in Fig. 5.7, the distribution of fruit weights differed between the two groups. The average fruit weight for WT (Azy) was 2.3 g. A small number of outliers were observed, including one notably heavier fruit weighing 10.1 g. In contrast, the mean FBPA fruit weight was 3.2 g, indicating an upward shift in fruit size relative to WT (Azy).

Overall, although both plant lines exhibited variability in fruit weight, FBPA overexpression was associated with fruit with higher average biomass in comparison to WT (Azy). Thus, reporting that FBPA overexpression has a positive impact on fruit weight.

5.4 Discussion

A total of eight FBPA overexpression lines were generated and screened, among which three lines (AW4-25, AW4-66, AW4-76, and AW4-80) were prioritised for further analysis based on stable transgene integration and expression. PCR and qPCR confirmed successful increased expression of FBPA across these lines, though with variation in transcript abundance.

Physiological characterisation showed that fruit-localised photosynthetic parameters were not significantly altered by FBPA overexpression. Measurements of PSII efficiency, including Fv/Fm, ΦPSII, qP, and NPQ, were comparable to wild-type azygous controls, suggesting that upregulation of FBPA did not improve light-use efficiency in the fruit at the $p < 0.05$ level.

Sugar content and carotenoid concentration assays revealed a reduction in Brix/total dissolved solids (primarily glucose, fructose, and sucrose) in the FBPA genotype. However, no statistically significant differences were observed for either lycopene or β-carotene concentrations. On the other hand, fruit biomass was increased in the FBPA Genotype.

Overall, the results demonstrate that FBPA overexpression in tomato fruit can alter carbon allocation, favouring increased fruit biomass at the expense of soluble sugar accumulation, without substantially affecting photosynthetic performance or carotenoid biosynthesis.

5.4.1 FBPA Does Not Impact Fruit Photosynthetic Efficiency

The absence of significant differences for all measured parameters in leaf photosynthesis between the FBPa-overexpressing tomato genotype and the WT (Azy) genotype suggests that the fruit-specific promoter, All-Flesh Fruit (AFF), used in this study functioned as intended and reported by Liu *et al.* (2022). It can be argued that AFF allowed for FBPA overexpression in fruit while leaving leaf photosynthetic mechanisms unaffected. However, further studies

involving leaf transcriptome and proteomic analysis are required to increase confidence in the construct not being expressed in foliar tissue.

That being said, I can be reasonably confident the transgene had little to no effect on leaf photosynthesis, as the constitutive overexpression of photosynthetic enzymes has frequently resulted in altered leaf metabolism and resource allocation (Miyagawa et al., 2001; Raines, 2003). The unaltered leaf photosynthetic performance in the FBPA lines, therefore, provides an important insight towards indicating promoter specificity and experimental design success.

Restricting FBPA overexpression to only fruit tissue is advantageous because it allows metabolic enhancement within the developing fruit without compromising source capacity in the leaves. By ensuring that this effect is localised to the sink organ, the plant can avoid potential competition between fruit and leaf tissues for photosynthetic intermediates or nitrogen resources.

The confirmation that leaf photosynthetic rates remain unaffected also provides a critical baseline for interpreting downstream fruit phenotypes. As a result, this finding increases confidence in the possibility that any observed increases in sugar content, nutritional value, or fruit size can be largely attributed to metabolic changes within the fruit, rather than to altered CBB cycle dynamics within the leaves.

The consistency between the 1-hour and 24-hour dark adaptation results suggests that the duration of dark adaptation does not significantly affect the results in fruit tissues, and that FBPA overexpression does not change the ability of fruit chloroplasts to utilise absorbed light energy. The subtle differences present following the 1-hour dark adaptation, where FBPA fruit exhibited slightly lower Fv'/Fm' values at intermediate irradiances, suggest that FBPA overexpression subtly shifts the photochemistry under specific light conditions. Such a response could reflect minor adjustments in the balance between excitation pressure and downstream metabolism. The absence of corresponding changes in Fq'/Fm', NPQ, or qP supports the conclusion that these effects are minor and do not compromise overall energy partitioning or photoprotective capacity. Instead, they highlight the robustness of the fruit's photosynthetic system and suggest that FBPA overexpression may exert only nuanced influences on PSII function while leaving broader light-use dynamics unchanged.

The carotenoid extraction assay result supports the observation that FBPA overexpression does not enhance fruit metabolism. The lack of difference in carotenoid concentration between the genotypes suggests that there were no differences in carbon fixation, which was observed. Studies have shown that improvements in photosynthesis can have a positive impact on carotenoid production. (Haldimann, 1996; Dhami, Tissue and Cazzonelli, 2018; Rodríguez *et al.*, 2018; Dannehl *et al.*, 2021).

Overall, the PSII efficiency results imply that FBPA overexpression does not enhance fruit photochemistry and may potentially hinder the partitioning of carbon into soluble sugars. This disconnect between photosynthetic capability and metabolic output highlights the significance of tissue context when considering modifications to CBB enzymes. In fruits, which primarily serve as heterotrophic sinks depending on imported photoassimilates (Blanke & Lenz, 1989; Carrara et al., 2001), enhancing a single step in the CBB cycle seems inadequate to improve photosynthetic contributions or metabolic yield.

In conclusion, FBPA overexpression in tomato fruit does not significantly alter PSII efficiency, photochemical quenching, or energy dissipation, indicating that the photochemical machinery of fruit chloroplasts remains largely unaffected. These results suggest that, in heterotrophic or partially autotrophic fruit tissues, photosynthetic capacity is primarily constrained by metabolic and carbon availability rather than by the activity of FBPa. The observed minor changes in Fv'/Fm' at intermediate irradiances emphasise that FBPA overexpression can subtly influence light-use dynamics without disrupting overall photochemistry. In summary, these findings emphasise that improving fruit photosynthesis may require the coordinated targeting of multiple CBB cycle enzymes, carbon partitioning pathways and possibly the introduction of genes involved in increasing the ability of carbon within the fruit rather than the isolated manipulation of a single enzyme.

5.4.2 The Divergent Role of FBPA in Source vs. Sink Tissues

Prior evidence shows that FBPA silencing in seedlings can reduce photosynthetic carbon metabolism and reduce tolerance to low temperature stress. This is likely due to its key role in maintaining triose phosphate availability and preventing feedback inhibition of photosynthesis (Cai *et al.*, 2018). These findings suggest that elevated FBPA activity could support sustained photochemistry under chilling stress by enhancing metabolic flexibility and preventing the over-reduction of electron transport components.

Despite this mechanistic rationale, overexpression of FBPA in fruit had no measurable impact on chlorophyll fluorescence parameters following chilling. There are three plausible explanations for this result. First, the role of FBPA during chilling may be highly context dependent, as it may have a stronger impact on actively growing vegetative tissues, which specialise as source tissues, rather than on ripening fruit, which is a sink tissue. The metabolic flux in these tissues differs, as does the carbon partitioning and carbon availability (Osorio, Ruan and Fernie, 2014; Colombié *et al.*, 2015). Second, fruit photochemistry under chilling may be limited more by thylakoid membrane integrity, reactive oxygen species (ROS) accumulation, or plastid lipid phase transitions than by the capacity of CBB cycle enzymes, meaning that FBPA overexpression cannot mitigate these dominant constraints (Miyake, 2020; Wójtowicz *et al.*, 2021; Zhao *et al.*, 2022). Finally, the chilling experiment may not have been severe or prolonged enough to reveal subtle differences in tolerance.

The absence of a measurable effect does not entirely rule out the possibility that FBPA may not have a role in improving fruit chilling tolerance. Still, it suggests that its contribution may be more relevant in combination with other metabolic or structural traits. Future work could investigate whether FBPA overexpression affects other chilling-related parameters in fruit, such as membrane fluidity, ROS scavenging, or carotenoid retention, and whether co-engineering with pathways related to energy dissipation or antioxidant capacity results in benefits.

While FBPA lines exhibited a modest increase in fruit fresh weight, there was no increase in either carotenoid content or sugar concentration. My findings suggest a partial decoupling of fruit biomass accumulation from the deposition of these primary and secondary metabolites, with important implications for both metabolic flux control and fruit quality.

Carotenoids, particularly lycopene and β-carotene, are critical determinants of tomato fruit nutritional value, pigmentation, and ripening (Bramley, 2002; Soytong *et al.*, 2021; Tufail *et al.*, 2024). These pigments accumulate during the transition from chloroplasts to chromoplasts and are tightly regulated by both developmental and metabolic cues (Sun *et al.*, 2018; Sadali *et al.*, 2019). Despite a statistically significant increase in fruit weight in FBPa-overexpressing lines, neither lycopene nor β-carotene levels differed significantly from wild-type azygous (WT (Azy)) controls. This result is noteworthy, considering previous research has shown that modifying CBB cycle enzymes can, in certain cases, lead to changes in metabolism and/or redox balance (Miyagawa, Tamoi and Shigeoka, 2001; Uematsu *et al.*, 2012; Cai *et al.*, 2022).

Overexpression of FBPA could potentially enhance the availability of glyceraldehyde-3-phosphate and pyruvate precursors that feed into the methylerythritol phosphate (MEP) pathway, which provides building blocks for carotenoids (Quian-Ulloa and Stange, 2021). However, the absence of a measurable change in carotenoid levels indicates that either the MEP pathway is not limited by substrate in ripe fruit under these conditions, or carotenoid biosynthesis is developmentally constrained and does not respond to moderate alterations in central carbon flux, or FBPA expression in fruit tissues is not sufficiently high to influence isoprenoid precursor pools. It is also possible that the spatial or temporal expression of FBPA did not significantly overlap with carotenoid biosynthesis, which primarily occurs in chromoplasts during the later stages of ripening (Kapoor *et al.*, 2022). Without an increase in the metabolic or transcriptional demand, the enhanced availability of triose phosphates may have been exported, diverted into other pathways, or remained underutilised.

Importantly, the lack of change in carotenoid concentration supports the idea that this trade-off is specific to primary carbon partitioning rather than a generalised shift in plastidial metabolism. The fact that fruit-localised photosynthetic capacity was unchanged between genotypes further suggests that the observed phenotypes arise from altered carbon utilisation rather than increased assimilation. This supports the notion that fruit sink metabolism, rather than local photosynthesis, is a critical determinant of fruit growth and quality. While FBPA has the potential to redirect carbon flux internally, it cannot overcome developmental or structural bottlenecks that constrain pigment accumulation.

In contrast to the unaltered carotenoid profile, total dissolved solids concentration (Brix) was significantly lower in FBPa-overexpressing fruit compared to WT (Azy). While the overall sugar levels remained within a typical range for tomato (5.2 to 8.7%) (Helyes, Pék and Lugasi, 2008), the reduction in Brix is particularly relevant from an agronomic and consumer quality perspective, as it directly affects sweetness and flavour perception. This decline in Brix, despite an increase in fruit biomass, suggests a dilution effect. This involves an increase in water or biomass, resulting in a lowering of the concentration of soluble sugars per unit fresh weight, a phenomenon commonly encountered in breeding programs that prioritise size or firmness (Cirilli, Bassi and Ciacciulli, 2016; Cakpo *et al.*, 2020). Such effects have also been previously observed in transgenic lines with altered sink size or aquaporin expression (Beauvoit et al., 2014). However, it is also plausible that FBPA overexpression redirected the assimilated carbon away from soluble sugar pools and toward other processes, such as the synthesis of structural

carbohydrates (cell wall polysaccharides), storage starch, or organic acids, particularly during early fruit development when growth is most active (Ripoll *et al.*, 2019; Mauxion, Chevalier and Gonzalez, 2021).

This altered carbon partitioning may have resulted from changes in the balance of glycolytic and gluconeogenic fluxes, potentially mediated by the FBPa-catalysed reaction and its influence on downstream metabolic processes. Additionally, FBPA overexpression may have shifted the equilibrium of carbon flux toward biosynthetic rather than osmotic functions, especially if other glycolytic enzymes are not co-upregulated. As a result, the observed reduction in Brix might reflect both physical dilution and metabolic reallocation.

An alternative or complementary explanation is that fruit sugar unloading, phloem transport efficiency, or vacuolar storage capacity may have been inadvertently affected by changes in sink strength. It is well-established that sugar accumulation in tomato is governed not only by source strength and photosynthesis, but also by the expression of sugar transporters, invertases, and vacuolar transporters, many of which are developmentally regulated (Ruan et al., 2012). If FBPA overexpression altered sink activity without upregulating these sugar-handling components, sugar import and storage efficiency may have lagged behind the expansion of biomass.

The overexpression of FBPA resulted in a statistically significant increase in ripe fruit fresh weight ($p = 0.0089$), which suggests that FBPA has a positive role in controlling fruit biomass. Interestingly, this increase occurred despite no detectable differences in fruit-localised photosynthetic capacity, carotenoid content and soluble sugar concentration. This suggests that FBPA overexpression causes a complex and uncoupled regulation of carbon partitioning, storage, and fruit quality traits.

As stated, FBPA overexpression in leaves has been linked to increased photosynthetic capacity and improved growth and biomass (Miyagawa, Tamoi and Shigeoka, 2001; Uematsu *et al.*, 2012; Cai *et al.*, 2022). One can argue that the significant increase in fruit biomass more likely reflects fruit-specific FBPA overexpression, which causes a shift in carbon utilisation or metabolic reprogramming within the fruit. FBPA may enhance the metabolic flux through glycolysis or the Calvin-Benson-like pathways that operate in fruit plastids, thereby supporting anabolic processes such as starch biosynthesis, cell wall formation, or organic acid production. These processes contribute to fruit expansion and biomass accumulation, independently of net photosynthetic input (Carrera *et al.*, 2021; MacNeill *et al.*, 2017; Batista-Silva *et al.*, 2018;

Quinet *et al.*, 2019; Cosgrove, 2022). It is also possible that FBPA activity enhances the capacity to process imported sucrose, improving the efficiency of sink metabolism during early fruit development. However, this hypothesis remains to be tested through sugar flux and starch content analyses.

Importantly, while the FBPa-overexpressing fruits had more mass on average, the distribution of fruit weights was notably broader and included several high-mass outliers. This suggests increased heterogeneity among individual fruits, which could result from variable transgene expression, altered developmental timing, or differential allocation of assimilates among fruit. Whether these changes reflect consistent physiological changes or arise from stochastic developmental effects remains to be determined.

In summary, fruit-specific overexpression of FBPA in tomato led to a significant increase in fresh fruit weight, independent of changes in fruit-localised photosynthetic capacity. This biomass gain was not accompanied by enhanced carotenoid accumulation and was negatively associated with soluble sugar content, indicating a trade-off between yield and quality. These findings suggest that FBPA modulates fruit sink metabolism or carbon partitioning rather than enhancing local photosynthetic input, offering both opportunities and caveats for future metabolic engineering aimed at improving fruit yield and composition. Future studies could benefit from measuring additional parameters such as dry weight, starch accumulation, cell size/number, and transcriptomic changes to better resolve the mechanisms underlying FBPa-induced phenotypes. Additionally, from a metabolic engineering perspective, these findings highlight the need for a more coordinated, systems-level approach to improving fruit traits. Simply increasing the capacity of a single enzymatic step is unlikely to enhance nutritional or sensory quality unless it is matched by parallel changes in sugar transport, storage, and pigment biosynthesis. The use of combinatorial engineering strategies is likely required to achieve improvements in both yield and quality.

5.5 Conclusion

This chapter examined the effects of overexpressing FBPa, a CBB cycle enzyme involved in the regeneration of triose phosphates, on tomato fruit physiology and metabolism. Eight independent transgenic lines were generated, of which four (AW4-25, AW4-66, AW4-76, and AW4-80) were confirmed to exhibit stable transgene integration and expression. Molecular

analysis confirmed elevated FBPA transcript abundance, although the extent of overexpression varied among lines. Despite this, fruit-localised chlorophyll fluorescence parameters (Fv/Fm, ΦPSII, qP, and NPQ) were unaffected (as reported by the conventional threshold for statistical significance ($p < 0.05$)). This demonstrates that increased expression of FBPA did not result in enhanced photochemical efficiency or overall photosynthetic capacity in tomato fruit.

Biochemical profiling revealed that FBPA overexpression altered fruit metabolism in a manner that decoupled biomass accumulation from sugar content. While the Brix values were significantly reduced, fruit fresh weight increased. This suggests that carbon allocation was redirected away from sugar accumulation and towards either growth processes or water content. However, carotenoid levels remained unchanged, which indicates that the diversion of carbon fluxes did not extend to secondary metabolism within the carotenoid biosynthetic pathway.

These findings suggest that manipulating a single CBB cycle enzyme, despite its capability to reprogram carbon partitioning, is insufficient to overcome the inherent limitations of fruit photosynthesis. Unlike leaves, tomato fruit have restricted stomatal density and limited internal light penetration. Consequently, while FBPA overexpression shifted sink metabolism towards greater biomass accumulation, it did not improve the fundamental constraints on photosynthetic performance in the fruit.

Together with the data presented in earlier chapters, the results highlight a broader principle: single-gene manipulations of CBB cycle enzymes in fruit provide only modest or context-dependent benefits. SBPase manipulation reduced photosynthetic capacity, and CAO expression impacted light-harvesting efficiency. FBPA overexpression primarily influenced carbon partitioning without improving photosynthetic traits. These outcomes underline the complexity of fruit photosynthesis, where improvements are unlikely to arise from isolated changes but instead may require coordinated engineering of multiple metabolic nodes in concert with modifications to carbon transport and sink strength.

In conclusion, FBPA overexpression demonstrates that metabolic adjustments in the CBB cycle can influence fruit biomass and quality traits but are unlikely to provide a direct route to enhanced fruit photosynthetic capacity. Future strategies should therefore focus on integrating multiple enzymatic targets, alongside anatomical or transport modifications, to more

effectively enhance the contribution of fruit photosynthesis to tomato productivity and nutritional value.

5.6 Bibliography

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6 Chapter 6: Regulating Chlorophyll a Oxygenase (CAO) In Fruit to Explore Fine-Tuning Light Capture Ability and Increasing Photosynthetic Rate

6.1 Introduction

A plant's light-capturing ability is equally important as the molecular processes that comprise the photosynthetic process. Thus, it can be said that light-capture ability is directly related to crop yield, quality and rate of development (Makino and Mae, 1999; Zhu, Long and Ort, 2010; Irigoyen et al., 2014). There are numerous benefits to improving both light capture ability and photosynthetic capacity in crop plants. These benefits include improving global food security, nutritional value, and overall quality (M *et al.*, 2024; Zhang *et al.*, 2025; Simkin, López-Calcagno and Raines, 2019).

There are numerous experimental methods which one could explore to increase the net photosynthetic capacity (Simkin *et al.*, 2015; Simkin *et al.*, 2020). One such method includes improving the photosynthetic capacity of fruit through differentially expressing genes involved in the light-harvesting apparatus, such as the antenna complex and chloroplast size or number (Croce *et al.*, 2024). One specific method includes using molecular biology techniques to differentially express a gene called Chlorophyll A Oxygenase (CAO) (Biswal *et al.*, 2024; Pattanayak *et al.*, 2005; Yamasato *et al.*, 2005). This gene has the function of coding for an enzyme with the function of catalysing the reaction of converting the pigment chlorophyll *a* into chlorophyll *b* (Tanaka *et al.*, 1998; Li *et al.*, 2021). Chlorophyll *b* is an accessory pigment; its primary function is to harvest light energy and pass it on to chlorophyll *a* for further use (Nobel, 2009). These pigments are present in the antenna complexes within photosystem I and II (Tanaka *et al.*, 1998; Jia, Ito and Tanaka, 2016). As mentioned, these pigments compose part of the antenna complexes; their specific function is that they both absorb specific wavelengths of light. Chlorophyll a absorbs light at wavelengths between 430nm and 662 nm, whereas chlorophyll b absorbs light at wavelengths between 450nm and 650 nm (Guidi, Tattini and Landi, 2017). The use of molecular biology techniques to downregulate CAO can allow for

antenna complexes with a reduced optical cross-section (Biswal *et al.*, 2024; Xiong *et al.*, 2024; Croce *et al.*, 2024). This is a result of the ratio of chlorophyll *a* to chlorophyll *b* being directly related to the optical cross-section. By reducing the number of chlorophyll *b* molecules, the size of the cross-section can be reduced along with its ability to capture light (Tanaka *et al.*, 1998; Li *et al.*, 2021).

A reduction in the antenna complex's optical cross-section and thus its light-harvesting ability can be beneficial, especially in high-light environments. In such environments, in most plants, the light conditions are not the rate-limiting factor regarding photosynthesis; the electron transport chain and an increased rate of nonphotochemical quenching become the limiting factor (Croce *et al.*, 2024; Holt, Fleming and Niyogi, 2004). This is because there will be an excess of captured energy with no available useful method for its use or transport. Thus, this energy is lost as heat energy in a process called Nonphotochemical quenching (NPQ) (Holzwarth, Lenk and Jahns, 2013). If the light-harvesting ability of the antenna complexes is reduced in crops typically grown in high-light conditions, this will reduce the likelihood that an excess of energy is captured that can be effectively used, thus increasing efficiency (Tanaka *et al.*, 1998).

In addition to NPQ, shown to be reduced in leaf tissue by the downregulation of CAO, reducing the size of antenna complexes can also allow for a greater proportion of light to penetrate through the canopy (Tanaka *et al.*, 1998). In the case of fruit, I will investigate the downregulation of CAO, which will allow light to penetrate deeper into the cell layers within the pericarp. This could cause the light capture ability of each light-harvesting complex to cause an overall increase in light captured as more antenna complexes have access to photosynthetically active radiation (PAR).

6.2 Materials and Methods

6.2.1 Construct Design and Generation of Plant Material

The construct design and the growth conditions for the plant material can be found collated in the chapter titled "Transformation chapter". The full material methods section can be found in Chapter 2. For all results presented in this chapter, differences were considered statistically

significant at the conventional threshold of $p < 0.05$. For other physiological methods not described in this section, please see Chapter 3.

6.2.2 Fruit Localised Silencing of CAO

I have silenced CAO only in fruit tissue with the aid of a strong and consistent fruit-specific promoter called TFM7 (Davuluri *et al.*, 2005). This promoter is a useful tool for allowing a gene of interest to be strongly expressed in a targeted manner. TFM7 allows fruit-specific expression of a gene, specifically during the early stages of development.

It is possible to downregulate CAO with the aid of interfering ribonucleic acid (RNAi) (Koeppe, Kawchuk and Kalischuk, 2023). RNAi functions by utilising existing mechanisms within the plant to reduce gene expression via post-translational processes. More specifically, gene knockdown via RNAi works by producing a double-stranded RNA (dsRNA) molecule of a specific design (Mocellin and Provenzano, 2004; Sen and Blau, 2006). This dsRNA will be detected and processed by an enzyme called Dicer, a member of the RNase III family of endonucleases. This enzyme will cleave the dsRNA into small fragments (typically less than 30 nucleotides). These fragments are called small interfering RNAs (siRNAs). siRNAs can act as guide strands once combined with a multiprotein complex called an RNA-induced silencing complex (RISC) (Muhammad *et al.*, 2019). The other strand produced from the digested dsRNAi is called the passenger strand; this segment is degraded.

The RISC protein, in conjunction with the siRNA guide strand, will seek the complementary sequences within mRNA. Once the guide strand has facilitated the binding of its attached RISC protein to a complementary region on an mRNA strand, RISC will cleave the mRNA and cause its degradation (Muhammad *et al.*, 2019; Kowsalya *et al.*, 2024; Liu *et al.*, 2021; Termolino, 2021).

6.2.3 SPAD

Leaf chlorophyll content estimations were carried out with the use of a portable chlorophyll meter (SPAD-502Plus, Konica Minolta, Japan). The meter was used to measure leaf greenness by determining the transmission of red (650 nm) and near-infrared (940 nm) light through the leaf. These values were used to calculate the SPAD value (unitless) that positively correlated with the relative chlorophyll concentration.

The SPAD meter was calibrated against the white standard provided before each sampling session according to the manufacturer's instructions. All measurements taken were in situ on young fully expanded leaves present 6 weeks after germination. 5 leaves per plant (two from the upper canopy, 2 from the middle, and one from the lower canopy) were selected at random. On each leaf, three readings were recorded at the central lamina, avoiding the midrib and any visible damage. The mean of these readings was recorded for each line. This physiological experiment was designed similarly to what was previously described in section 3.2.2 regarding replicates. Measurements were conducted under the growth conditions stated in section 2.2.12 between 11:00 am and 01:00 pm to minimise any diurnal variations.

6.2.4 Chlorophyll Extraction Assays

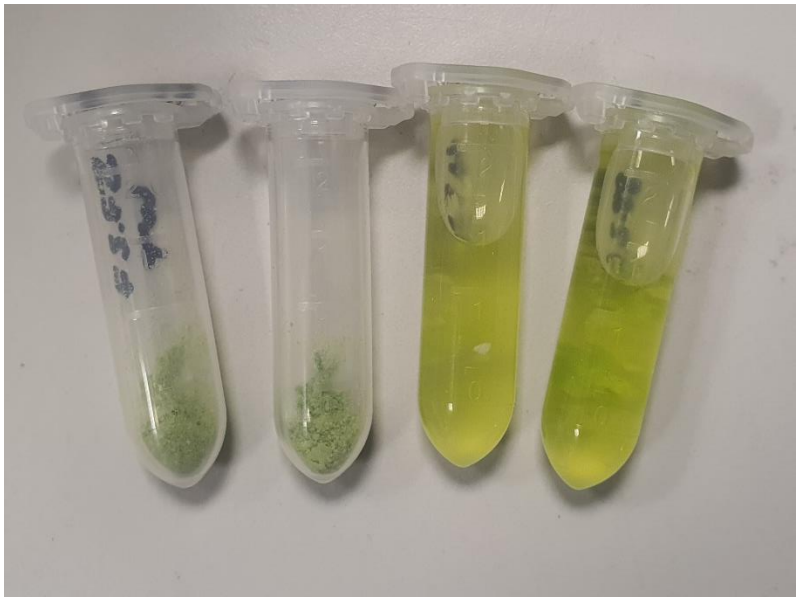

Figure 6.1. *The two 2mL Eppendorf tubes on the left-hand side contain freeze-dried unripe tomato fruit tissue. The tubes on the right are examples of freeze-dried tissue that has had its chlorophyll extracted in N,N-dimethylformamide (DMF).*

Chlorophyll extraction assays were conducted by picking fruit in the mature green stage, approximately 25-27 DPA (Fig. 2.15), and then freezing them in a -80 °C freezer for 24 hours before freeze-drying them for 48 hours. Upon freeze-dry completion, each fruit was ground into a fine powder with a pestle and mortar. The powder was then put into a 15 mL tube for storage. Upon use for chlorophyll extraction, 25mg of the powder was weighed out and placed into a 2mL Eppendorf tube, and then 2mL of Dimethylformamide (DMF), chilled to 4 °C, was

pipetted into the Eppendorf (Fig. 6.1). It was then covered with foil to prevent light from entering the tube. The tubes were then stored in a fridge for 24 hours.

After 24 hours had elapsed, the tubes were put into a centrifuge for 2 minutes at 13,000 RPM. Once completed, 1.5 mL of the solution was placed into a glass cuvette, and the absorbance values were measured at 665nm to quantify the concentration of chlorophyll *a* and 647nm to quantify the concentration of chlorophyll *b*.

The following equations were used to calculate the concentration of each chlorophyll pigment from the optical density (OD) measurements obtained:

The concentration of chlorophyll *a* (mg/mL) = (12 x abs at 665nm) – (3.11 x abs at 647nm)

The concentration of chlorophyll *b* (mg/mL) = (20.78 x abs at 647nm) – (4.88 x abs at 647nm)

Both equations were borrowed from Lupi *et al.* (2019).

6.2.5 Fruit Biomass Measurements

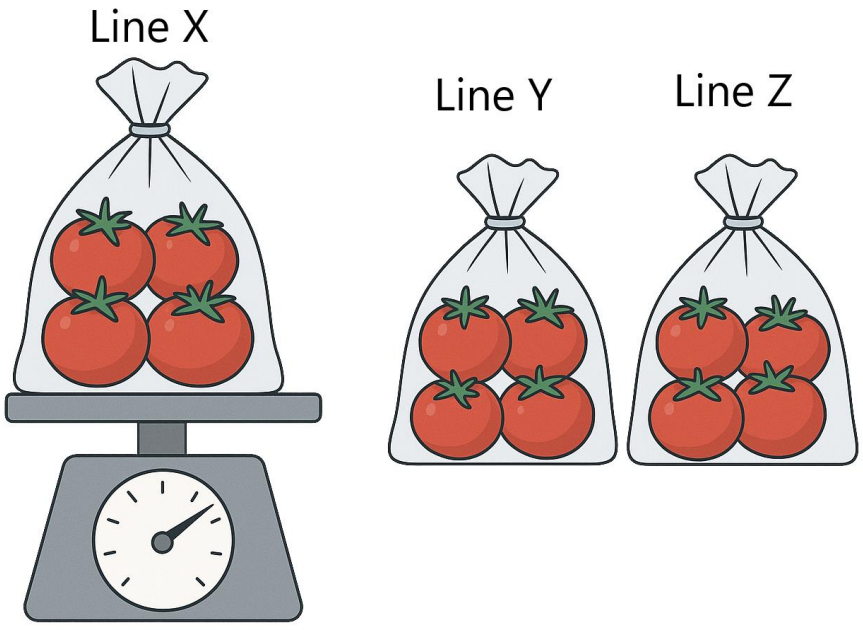

Figure 6.2. *Diagram illustrating the method used to measure fruit biomass. Fruits from each plant line (Lines X, Y, and Z, shown here as illustrative examples) were collected, weighed, and counted to calculate the average fruit biomass per genotype once pooled. (Diagram generated by ChatGPT (OpenAI) (Oct 2025))*

Fruit biomass measurements were conducted over a 3-month period. To ensure full ripening, fruits were marked at the early breaker stage (35–40 DPA (Fig. 2.15)) with a permanent marker

and harvested two weeks later. Harvested fruits were stored at 5 degrees Celsius in plastic bags, with fruits from the same plant being stored together. Each bag represented an independent biological replicate. In this way, the bag became the unit of measurement for assessing biological variability.

At the end of the collection period, each bag was weighed individually using a precision balance, and the total fruit biomass was recorded in addition to the number of fruits present. Results were then grouped by genotype (iCAO vs. wild-type (azygous)), and the total biomass for each genotype was divided by the total number of fruit. This provided an average fruit biomass for each biomass.

6.2.6 Results SPAD

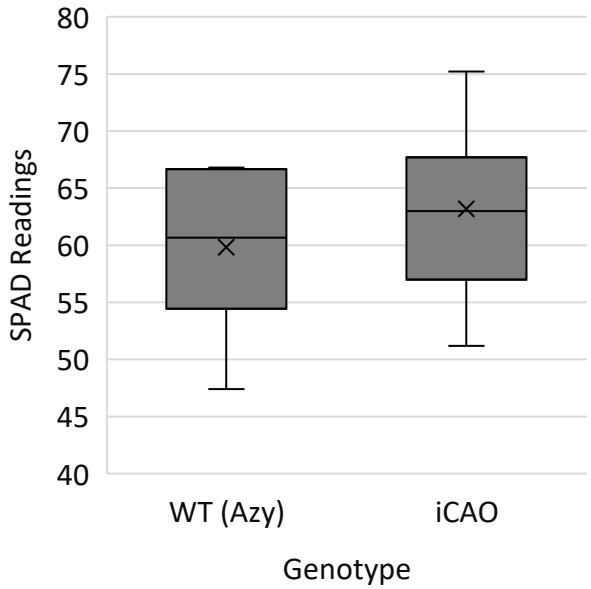

Figure 6.3. *A box and whisker plot showing the 5-leaf average SPAD readings for the WT (Azy) and iCAO genotypes. The WT (Azy) had an average of 59.82, and the iCAO fruit had an average of 63.19 ($p > 0.05$)*

SPAD measurements were used to estimate leaf chlorophyll content in WT (Azy) and iCAO plants. Each value represents the average of five leaf readings per plant. iCAO plants exhibited a slightly higher median and mean SPAD value compared to WT (Azy); however, the difference was not statistically significant (Fig. 6.4). Both genotypes showed overlapping interquartile ranges, with iCAO displaying a broader overall distribution. These results indicate that iCAO expression does not lead to a significant change in total chlorophyll content in the leaves under the conditions tested.

6.2.7 Chlorophyll Fluorescence Imaging

6.2.7.1 Leaf Tissue

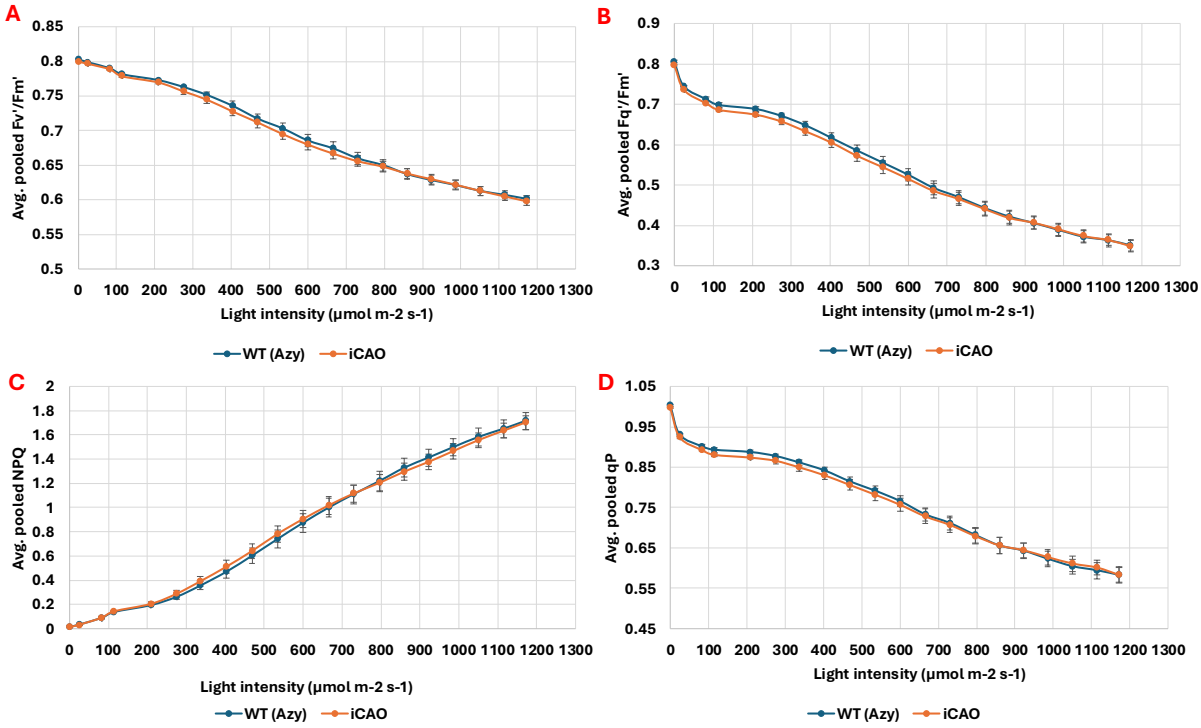

Figure 6.4. *Leaf fluorescence light response curves showing the* **(A)** *Fv'/Fm',* **(B)** *Fq'/Fm'/ΦPSII,* **(C)** *NPQ, and* **(D)** *qP. Results were obtained from the young, fully expanded leaves that were harvested and dark-adapted for 20 minutes before imaging. Data is shown as mean ± SD from pooled biological replicates (n ≥ 3). Blue lines indicate the WT (Azy) genotype; orange lines represent the iCAO genotype.*

The fluorescence light response curves (Fig. 6.5) conducted on the leaf tissue show that there was no statistically significant difference between the transgenic and the WT (Azy) genotype in any measurement under the conditions tested. More specifically, no statistical differences were observed in the key photosynthetic parameters: maximum quantum efficiency of PSII in a dark-adapted state (Fv/Fm) (show at 0 µmol m^{-2} s^{-1} within Fig. 6.5A), maximum quantum efficiency of PSII in the light (Fv'/Fm') (Fig. 6.5A), effective quantum yield (ΦPSII, or Fq'/Fm') (Fig. 6.5B), non-photochemical quenching (NPQ) (Fig. 6.5C), and photochemical quenching (qP) (Fig. 6.5D).

6.2.7.2 1 Hour Dark Adaptation of The Fruit

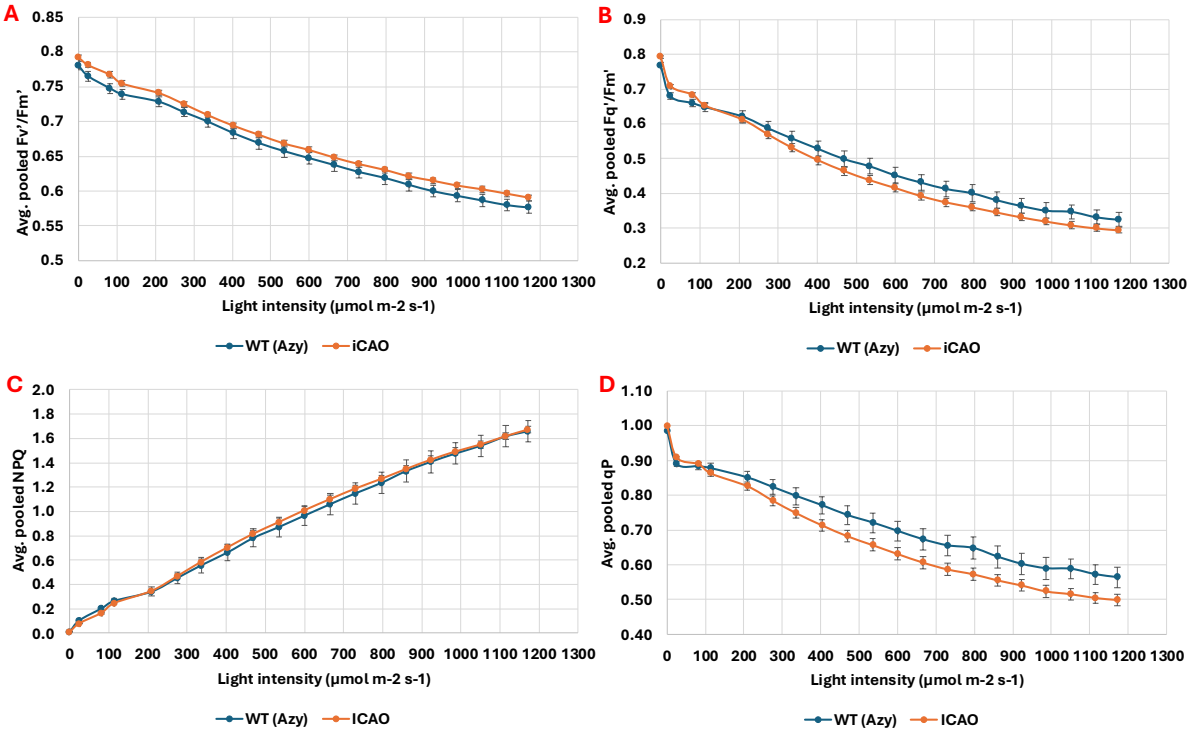

Figure 6.5. *Light response curves conducted on 1-hour dark-adapted unripe fruit:* **(A)** *Fv'/Fm',* **(B)** *Fq'/Fm' (also known as ΦPSII),* **(C)** *NPQ, and* **(D)** *qP. A total of 20 measurements were taken at increasing light intensities from 0 µmol m^{-2} s^{-1} to a maximum of 1172 µmol m^{-2} s^{-1} over approximately 60 minutes. Data is shown as mean ± SD from pooled biological replicates (n ≥ 3). Blue lines indicate the WT (Azy) genotype; orange lines represent the iCAO genotype.*

Regarding the Fv'/Fm' data (Fig. 6.6A), statistical differences were observed at the lower light intensities 25-82 µmol µmol m^{-2} s^{-1}. The Fv/Fm value, at 0 µmol m^{-2} s^{-1}, which represents the maximum potential quantum efficiency of PSII photochemistry in a dark-adapted state, showed near statistical significance ($p = 0.077$).

The Fq'/Fm' results (Fig. 6.6B) showed significance at the lower light intensities between 0 and 82 µmol m^{-2} s^{-1}, with iCAO outperforming the control. Similarly, the NPQ results (Fig. 6.6C) revealed a similar result with significant differences only being observed between 0 and 82 µmol m^{-2} s^{-1}. The qP data (Fig. 6.6D) suggest that WT (Azy) fruit performance begins to surpass that of the iCAO genotype at medium to high light intensities. This is first indicated by the borderline statistical significance observed between 403 and 1172 µmol m^{-2} s^{-1} ($p \approx 0.05$). However, a consistently significant difference is only noted at the three highest light intensities, indicating that the photosynthetic disadvantage of iCAO is most evident under strong irradiance. Throughout all measurements, the minimal variation shown by the error bars, which

represent standard deviation, suggests high reproducibility among both technical and biological replicates.

6.2.7.3 24-Hour Dark Adaptation of The Fruit

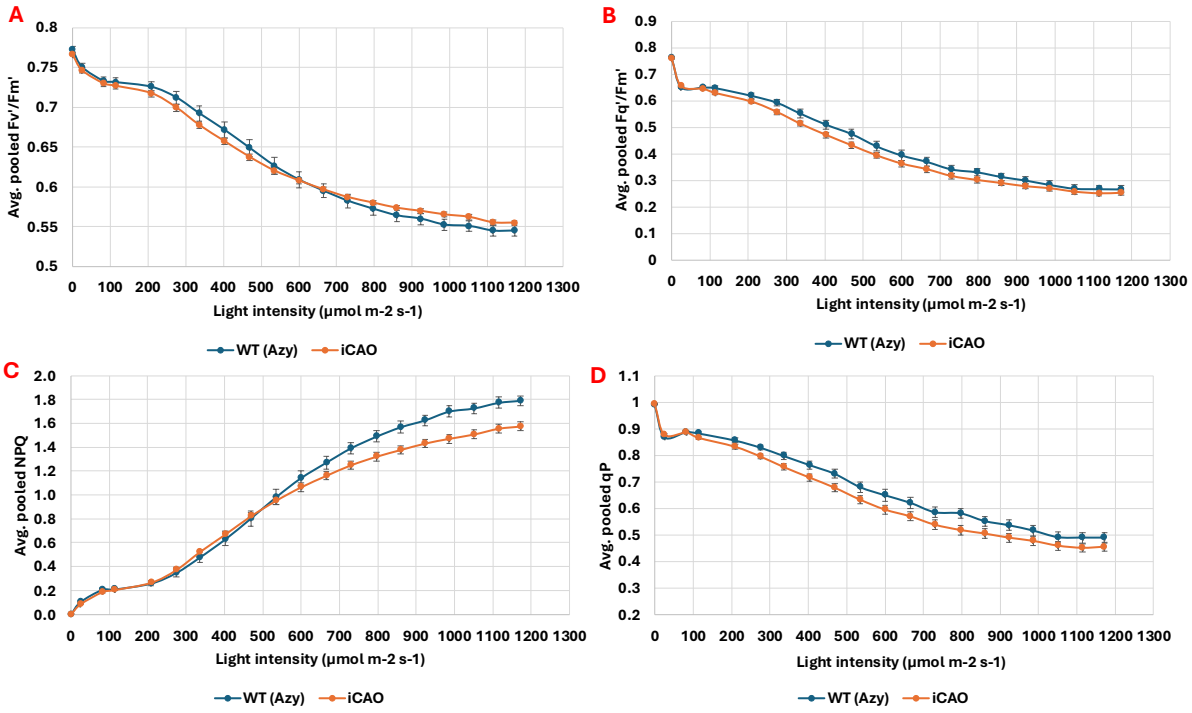

Figure 6.6. *A fluorescence light response curve showing the* **(A)** *Fv'/Fm', (B)Fq'/Fm'/ΦPSII,* **(C)** *NPQ, and* **(D)** *qP results that were obtained from the mature unripe fruit that was harvested and stored at 24 °C and in complete darkness for 24 hours before imaging. Error bars represent standard deviation. Data is shown as mean ± SD from pooled biological replicates (n ≥ 3). Blue lines indicate the WT (Azy) genotype; orange lines represent the iCAO genotype.*

After a 24-hour dark adaptation of the fruit, lower Fv'/Fm' values (Fig. 6.7A) were obtained compared to the 1-hour dark adaptation period experiment (Fig. 6.6A); however, no statistical differences were observed throughout. Concerning the Fq'/Fm' data (Fig. 6.7B), when comparing the data points from 275 to 469 µmol m^{-2} s^{-1}, p-values ranging from 0.045 to 0.061 were obtained. This indicates a significant difference between the treatments at this light intensity, with WT (Azy) outperforming the transgenic genotype.

The NPQ results obtained after a 24-hour dark adaptation (Fig. 6.7C) greatly differ from its 1-hour dark adaptation counterpart (Fig. 6.6C). The 24-hour dark adaptation data show that the transgenic and WT (Azy) lines respond similarly to light intensities up to 730 µmol m^{-2} s^{-1}; at and beyond this irradiance, significant differences were observed with the iCAO genotype

experiencing less NPQ. The qP result indicates that the iCAO genotype performed similarly to the WT (Azy), but with slightly lower values. Significance was observed at 403, 469 and 797 µmol m^{-2} s^{-1} (Fig. 6.7D) with a lower proportion of PSII reaction centres open in the iCAO genotype.

6.2.8 Pigment Content and Sugar Content by Brix Analysis

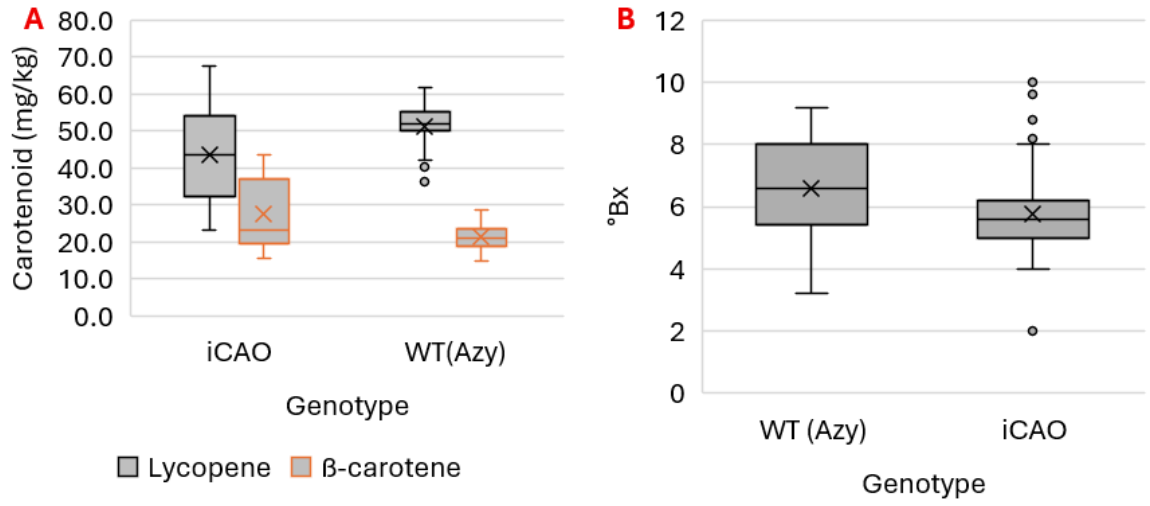

Figure 6.7. (A) Carotenoid content in WT (Azy) and iCAO tomato genotypes measured via extraction assay. *Boxplots show concentrations (mg/kg fresh weight) of lycopene (grey) ($p < 0.05$) and β-carotene (orange) ($p < 0.05$) in wild-type azygous and transgenic iCAO fruit.* **(B)** *A Box and Whisker plot showing the sugar content by Brix analysis data from both genotypes, transgenic and wild-type azygous ($p < 0.05$). The central line represents the median, boxes indicate the interquartile range, whiskers show the range excluding outliers, and crosses (×) denote the mean. Outliers are represented as individual points.*

The results show that lycopene was the most abundant carotenoid in both genotypes; however, iCAO fruit exhibited a wider range of lycopene concentrations (23.21–67.50 mg/kg), with a mean of 43.55 mg/kg (Fig. 6.8A). In contrast, WT (Azy) fruit displayed lycopene values ranging from 36.37 to 61.78 mg/kg, with a mean of 51.13 mg/kg. The difference in mean lycopene content between genotypes was statistically significant ($p < 0.05$), with WT (Azy) having a higher concentration.

In iCAO fruit, β-carotene ranged from 15.61 to 43.39 mg/kg, with a mean of 27.60 mg/kg. WT (Azy) fruit exhibited lower β-carotene concentrations overall, ranging from 14.82 to 28.45 mg/kg, with a median of 21.35 mg/kg and a mean of 21.35 mg/kg. The increase in β-carotene levels in iCAO fruit relative to WT (Azy) was found to be statistically significant, indicating that iCAO enhances β-carotene accumulation in fruit tissue.

The juice of 110 mature ripe iCAO fruit and 55 WT (Azy) ripe fruits was extracted and used to conduct a Brix measurement test, which indicated total dissolved solids (primarily glucose, fructose, and sucrose). As shown in Figure 6.4B, the results suggest that, on average, the WT (Azy) fruit contained a greater quantity of dissolved solids within the juice (6.63%) when compared to that of the transgenic fruit (5.76%). A statistical analysis revealed a difference between the averages for each genotype. This reports that with a high level of certainty, the WT (Azy) genotype contained more dissolved solids and thus dissolved sugars in its extracted juice (Fig. 6.4B).

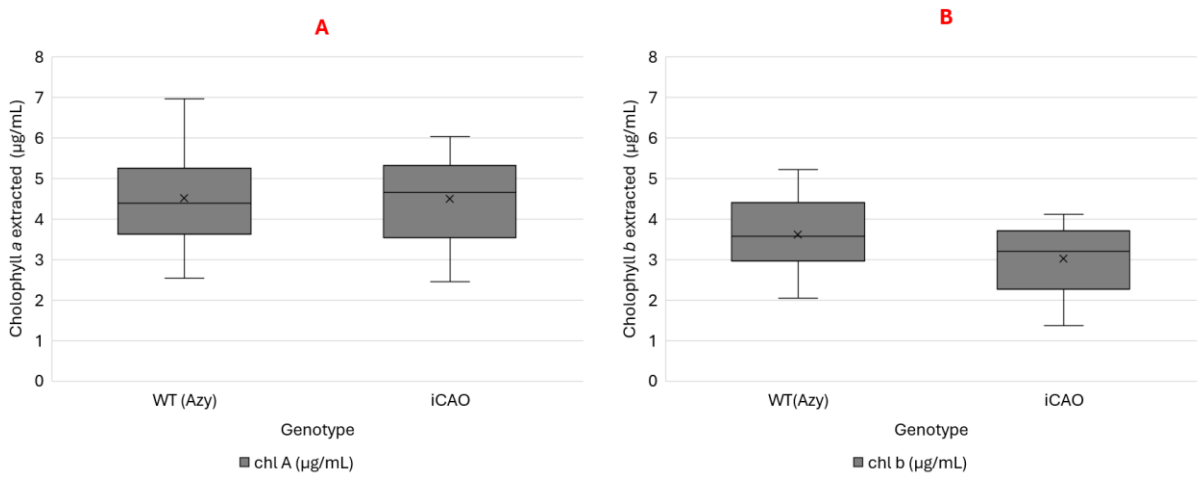

Figure 6.8. *Chlorophyll extraction data showing*: **(A)** *concentration of chlorophyll a (µg/mL) extracted from fruit ($p > 0.05$), and* **(B)** *concentration of chlorophyll b (µg/mL) extracted from fruit ($p < 0.05$).*

Chlorophyll content was quantified in WT(Azy) and iCAO lines to determine whether the genotype influenced pigment accumulation and composition (Fig. 6.9). Chlorophyll *a* concentration did not differ significantly between genotypes (Fig. 6.9A). By contrast, chlorophyll *b* levels were significantly lower in iCAO compared to WT(Azy) (Fig. 6.9B).

6.2.9 Fruit Biomass Measurements

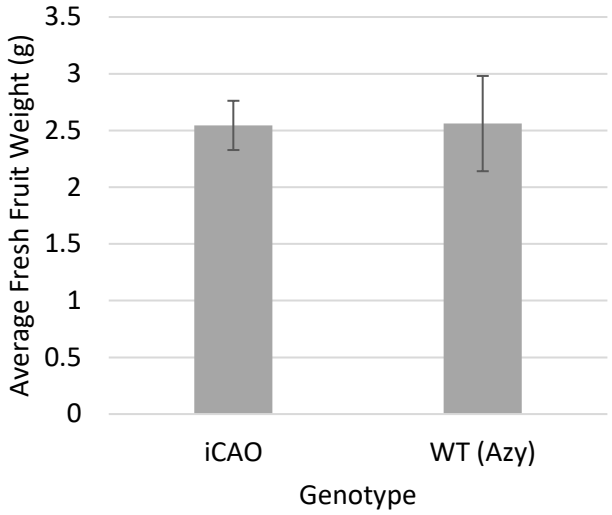

Figure 6.9. *The average weight of fresh, ripe fruit was pooled by genotype and then averaged ($p > 0.05$). Error bars represent standard deviation.*

The average fresh fruit biomass result shows a 0.02g difference between the iCAO and the WT (Azy) genotypes; however, no statistical significance was observed between the two groups.

6.3 Discussion

As expected from the fruit-specific nature of the construct, fruit-specific silencing of CAO did not alter leaf physiology, with SPAD values and fluorescence light response curves showing no differences compared with WT (Azy). In contrast, fruit tissues exhibited clear genotype-dependent effects. Chlorophyll fluorescence assays revealed that iCAO fruit displayed altered PSII performance, characterised by higher Fv'/Fm' at low irradiance, increased qP at saturating light, and reduced NPQ under strong irradiance. These shifts suggest that iCAO expression influences how fruit photosystems balance light use and photoprotection under variable light environments.

Metabolic profiling also highlighted fruit-specific changes in pigment and metabolite composition. iCAO fruit accumulated less lycopene but more β-carotene, contained lower soluble sugar levels, and showed a higher chlorophyll *a*/*b* ratio driven by reduced chlorophyll

b. Together, these results indicate that iCAO manipulation reshaped pigment partitioning and metabolic allocation in fruit without affecting vegetative tissues.

6.3.1 The Photosynthetic Benefits of Fruit-localised iCAO May Be Context-dependent

It was essential to determine whether any differences existed in leaf photosynthetic performance between genotypes. This was important as even a slight variation in the photosynthetic capability of the leaves could have a substantial impact on fruit photosynthesis. This data quells any concerns relating to the undesired translocation of the fruit-produced CAO siRNAs. As siRNAs can travel medium to long distances to distal organs within a plant, it was essential to ensure that they did not have a significant silencing effect on CAO gene expression in leaf tissue, as it could have an additive effect on the fruit physiology results (Brosnan and Voinnet, 2011). As mentioned, the leaf fluorescence imaging data indicate that this was not the case, as there was no significant difference between the averaged transgenic genotypes and the averaged WT (Azy). Additionally, the SPAD result aligns with what was anticipated, given that iCAO was expressed in fruit tissue. If iCAO was strongly affecting chlorophyll biosynthesis, I might expect a shift in SPAD values. However, the lack of a strong morphology here suggests that fruit iCAO expression is not affecting leaf chlorophyll biosynthesis. Thus, any differences between the genotypes are likely due to changes in fruit photosynthesis.

The comparative analysis of dark adaptation experiments conducted over 1 hour and 24 hours sheds light on the impact of CAO knockdown on photosynthesis in tomato fruit. The reduction of chlorophyll *b* levels can alleviate overexcitation after experiencing extended darkness (24 hours). My results suggest that a diminished chlorophyll *b* concentration does not improve excitation balance within PSII after a relatively short darkness adaptation period but rather limits the ability to keep reaction centres open during intense light exposure. The observed decline in qP after both dark adaptations correlates with a reduced functional antenna size, which hampers energy transfer efficiency (Bielczynski, Schansker and Croce, 2020). This points to the fact that changes in pigment composition may disrupt photochemical regulation in fruit tissue rather than optimise it.

After 24 hours of dark adaptation, the reduction in Fv/Fm and Fv'/Fm' values compared to the shorter dark adaptation period values suggests that extended periods of darkness may induce

mild stress on PSII, which was somewhat expected and likely due to the extended darkness causing partial degradation of pigments (Okada *et al.*, 1992; Weaver and Amasino, 2001; Shibaeva *et al.*, 2024). Regarding the high light intensities following a prolonged dark adaptation, the NPQ results imply increased thermal dissipation mechanisms. As this difference was not evident after 1 hour of dark adaptation, this indicates that changes to the antenna size result in context-dependent changes to the photochemical and photoprotective balance.

The variations identified between the two dark adaptation periods may stem from the degradation of pigments, as well as a reduction in the functional population of light-harvesting complex II (LHCII) after 24 hours (Okada et al., 1992; Weaver and Amasino, 2001; Shibaeva et al., 2024). The extended darkness likely provided more opportunity for a greater number of LHCIIs within the iCAO genotype to degrade. The degradation was likely faster in the transgenic genotype as reductions in chlorophyll *b* have been linked to the instability of LHCII (Eggink, Park and Hoober, 2001; Kim *et al.*, 2009; Sato, Ito and Tanaka, 2015).

Overall, the observed general decline in Fq'/Fm' for both treatments suggests that CAO knockdown does not lead to a consistent advantage in photosynthesis in tomato fruit. While some aspects of PSII efficiency were preserved, the anticipated benefits regarding excitation balance were not realised.

The findings suggest that structural changes to the antenna complex alone cannot mitigate the intrinsic metabolic and anatomical constraints on fruit photosynthesis. The inherent limitations of CO_2 diffusion and the carbon metabolism in tomato pericarp tissues indicate that the main bottleneck lies in the utilisation of absorbed energy, rather than light capture. Additionally, the comparison of different dark adaptation periods underscores the significance of previous light exposure and PSII acclimation on overall efficiency. Although modifying antenna structures can subtly influence photochemical behaviour, the complete advantages of such changes will only be realised when they are paired with approaches that enhance carbon assimilation and electron sink capacity. This could involve strategies such as boosting CBB cycle activity, adjusting source-sink dynamics, or increasing chloroplast development in fruit tissues.

Future research could expand on these insights by exploring the varying expression of CAO at different developmental stages and under diverse environmental stresses. For instance, studies on rice mutants have demonstrated that CAO knockouts experience reduced photosynthetic

performance under chilling conditions compared to wild-types (Xiong et al., 2024). Investigating whether similar sensitivities occur in tomato fruit could yield important insights into the wider physiological trade-offs associated with altering chlorophyll composition. Such research will help ascertain whether CAO knockdown could be beneficial for crop improvement or if its effects are too reliant on specific contexts to be particularly useful in agriculture. Additionally, future studies should involve methods that investigate whether the silencing of CAO in fruit improves the penetration of light through the upper fruit layers, as was assumed (Friedland *et al.*, 2019). This could provide further context towards the results observed, as chlorophyll fluorescence imaging does assess this metric.

6.3.2 iCAO Adjusts Carotenoid Partitioning and Reduces Sugar Accumulation

As a result of the chl extraction assay indicating that the transgenic fruit had a lower average concentration of chl b ratio compared to the WT (Azy), it can be inferred that the inhibition of CAO was successful. This allows me to confidently state that the hypothesis that the manipulation of the antenna size within fruit chloroplasts is possible and is a viable method to explore the manipulation and improvement of fruit photosynthetic apparatus.

Interestingly, the results still report the presence of chl *b* in notable concentrations in the loss-of-function mutants, albeit in lower concentrations than WT (Azy). This highly suggests that the conversion of chl *a* to chl *b* is occurring to some degree. Notably, despite the difference in chlorophyll ratio, there was no observable difference in visual phenotype. One might expect the hue of the fruit in an immature stage to differ from a WT (Azy) in the same stage, but this was not observed.

The evidence that the conversion is still taking place could suggest that an additional pathway exists for converting chl *a* to *b*. Alternatively, although the qPCR data reported an average reduction equal to 99.7% in the expression of CAO, a significant quantity of the CAO enzyme could still have been produced. Further experimentation could involve the use of protein assay studies, such as Western blot, to aid in understanding why the production of chlorophyll *b* remains.

A similar result was found in a study conducted by Biswal *et al.* (2024). This study involved the constitutive differential expression of CAO in tobacco plants; the CAO loss-of-function mutants still showed a significant production of chl *b,* but at lower concentrations than the WT. This result could have been obtained as a result of similar processes that took place within the fruit in this study. The function of this unknown process seems to prevent the tissue from being completely dominated by chl *a.* Research involving a construct that causes the chl ratio to be altered in favour of chl *b* would yield interesting results, as it would shed some light on whether this undescribed balancing mechanism can also work to prevent the complete lack of chl *a*.

The carotenoid profiles I observed indicate a shift in the carotenoid biosynthetic pathway's activity. My findings suggest that the decrease in Chl *b* and the resulting changes in the photosynthetic antenna's composition and stability could have downstream effects on metabolic flux, potentially redirecting precursors or energy away from lycopene synthesis. The wider range of lycopene concentrations in iCAO fruit also suggests that the effect of CAO knockdown is variable, possibly reflecting differences in local metabolic capacity or light capture within individual fruits.

These particular findings show that strategically altering chlorophyll composition leads to a significant consequence related to secondary metabolism, especially in carotenoid partitioning. The differing effects on lycopene and β-carotene highlight the relationship between light-harvesting efficiency, photosynthetic performance, and metabolic resources. This change could indicate a change in substrate availability or variations in enzymatic activity. This could be due to the modified light environment within the fruit as a result of the reduction of Chl *b*. As β-carotene functions as a protective pigment and a precursor for other beneficial metabolites (Tanno et al., 2020; Xie et al., 2020; Mathews-Roth, 1987), its increased accumulation may indicate its use to optimise energy use and protect the photosynthetic apparatus under the new light conditions within the fruit due to iCAO.

These particular findings show that strategically altering the chlorophyll balance can lead to a significant consequence related to secondary metabolism, especially in carotenoid partitioning. The differing effects on lycopene and β-carotene highlight the relationship between light-harvesting efficiency, photosynthetic performance, and metabolic resource allocation. CAO knockdown could be used in an industrial setting to tailor nutritional and photoprotective profiles of fruits. Future research that combines iCAO with the genetic engineering of

downstream carotenoid enzymes could further optimise the accumulation of specific carotenoids, thus further tailoring nutritional quality towards a specific goal.

The average Brix values, which indicate the concentration of soluble sugars and are commonly used as a proxy for fruit sweetness and quality in industrial settings (Jaywant, Singh and Arif, 2022), indicate that the iCAO construct negatively impacts sugar accumulation in the fruit. This result was largely unexpected, as both fruit and leaf chlorophyll fluorescence imaging showed no significant differences in photosynthetic capacity and efficiency at 400 µmol m^{-2} s^{-1}, which was the light intensity the plants experienced throughout the day. A possible explanation for this result involves the chlorophyll fluorescence parameters recorded. Fv'/Fm' and Fq'/Fm' primarily measure efficiency rather than capacity. In other words, while the photochemical function of PSII remains intact in iCAO, the fruit, the total photon absorption over time may be reduced due to the decreased size of the LHC and lower chlorophyll content. This would likely cause a reduced daily light-harvesting capacity and, therefore, a small but measurable decrease in carbon assimilation, even if PSII function appears unaffected.

Another possibility is that the reduction in sugars arises from sink-localised effects within the fruit itself. Chlorophyll *b* plays a key role in the maintenance of the structure and function of plastids in green tissue (Schwarz and Kloppstech, 1982; Eggink, Park and Hoober, 2001; Hoober, Eggink and Chen, 2007). During fruit development, chloroplasts are metabolically active and contribute to early-stage photosynthesis and carbon fixation. Reducing CAO expression may decrease chloroplast integrity or metabolic function and thus reduce the fruit's capacity to produce and store sugars. Additionally, a reduction in chlorophyll *b* concentration may alter plastid-to-nucleus signalling by interrupting the complex gene network involved in sugar metabolism and/or transporter expression.

It is also possible that source-to-sink carbon allocation is affected. If iCAO fruits have lower sink strength due to reduced plastid development or altered hormone signalling, they may import less sugar from photosynthetic tissues, despite those source tissues functioning effectively. Such shifts in carbon allocation would not be detected through chlorophyll fluorescence measurements but still could significantly influence fruit sugar content (Bihmidine *et al.*, 2013; Nakai *et al.*, 2024).

Finally, a broader biological context should be considered as fruit sugar accumulation is controlled by a complex relationship between photosynthesis, translocation, metabolism, and

development (Chen et al., 2021; Ren, Liao and Xu, 2023; Wu et al., 2025). The reduced Brix phenotype observed in iCAO fruit, despite unaltered PSII efficiency, stresses the limitations of primarily relying on chlorophyll fluorescence imaging when assessing whole-plant carbon economy and highlights the importance of integrating metabolic and developmental data. However, this was unavoidable due to the fruit's lack of stomata and financial constraints relating to the design of experiments that could provide a biologically holistic view.

Overall, my result shows that silencing CAO does not significantly affect fruit biomass, and development is somewhat unexpected, given that I hypothesised that iCAO might influence the chlorophyll partitioning during fruit development and potentially alter photosynthetic activity by allowing more reaction centres present within the deeper layers of the fruit tissue to be able to harvest more PAR. I hypothesised that as more reaction centres are in play, the increased energy capture could theoretically improve carbon accumulation or growth rates within the fruit, thereby influencing final biomass.

However, when considering the chlorophyll fluorescence imaging data, it has been shown that under the specific light conditions used, there were no meaningful photosynthetic benefits from the reduction of CAO expression. Therefore, the fact that there was no difference in fruit biomass between the genotypes is expected, as there was no difference in photosynthesis. One could expect to see differences in biomass if the plants were grown in conditions with a light intensity above 800 μmol m^{-2} s^{-1} and large fluctuations for extended periods. This is because these conditions will likely trigger higher rates of NPQ in the fruit, which the iCAO genotype is less vulnerable to.

6.4 Conclusion

The fruit-specific knockdown of CAO provided a novel insight into how changes to chlorophyll biosynthesis can impact fruit photosynthesis and metabolism. My findings suggest that iCAO manipulation can be used as a means to influence carotenoid concentration, as iCAO fruit accumulated more β-carotene at the expense of lycopene, thus revealing a shift in carotenoid partitioning. This shift highlights that there is a close relation between the chlorophyll and carotenoid biosynthesis pathways. From a nutritional perspective, a method to increase β-carotene, a provitamin A compound, could be a valuable and viable method to achieve biofortification and combat vitamin A deficiency. From an industrial perspective, this

partitioning may be advantageous in applications that require high β-carotene content. Future work could refine this approach by exploring developmental stage-specific promoters or combining iCAO manipulation with other pathway genes to tailor carotenoid composition for consumer health and industrial demand.

Additionally, contrary to expectations that reduced CAO expression would increase PAR penetration and thus increase net photosynthetic efficiency and sugar accumulation, iCAO fruit showed lower Brix without a loss in biomass. It can be argued that this indicates that CAO manipulation alters metabolic partitioning rather than overall growth.

Overall, this study showed that modifying chlorophyll biosynthesis via iCAO can have a measurable and targeted effect on carotenoid concentration, PSII function (under specific conditions), and downstream metabolic traits. The results report that fruits are unique regulatory environments, in which reductions in chlorophyll *b* concentration not only can affect photosynthetic properties but also can influence carotenoid biosynthesis and reduce sugar accumulation without shifting carbon towards biomass accumulation.

Future work should involve the analysis of the mechanism or mechanisms that link the altered pigment composition to the observed downstream metabolic shifts. This would be necessary to help clarify whether the reduction in sugar and lycopene reflects a direct limitation on fruit photosynthetic capacity, or if it is due to an alteration in signalling between photosynthetic and metabolic pathways. These questions are crucial to a complete understanding of how fruit photosynthesis contributes to yield and nutritional quality. Furthermore, further analysis of the mechanisms at play may aid in the telling of whether the targeted manipulation of chlorophyll metabolism in this manner should continue to be exploited for crop improvement. In addition, future experiments could grow the mutant plant under high-light conditions (> 750 μmol m^{-2} s^{-1}). This may yield interesting results, as this may give the iCAO fruit more of an opportunity to reveal its theoretically improved ability to resist higher NPQ values.

6.5 Bibliography

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Chapter 7: General Discussion

6.6 Summary of Key Findings and Research Aims

Although NFP is often overlooked regarding crop improvement, it is becoming increasingly recognised as contributing towards sugar accumulation and nutritional quality (Simkin *et al.*, 2020). Through the production and characterisation of transgenic tomato lines with fruit-specific overexpression or knockdown of SBPase, FBPa, and CAO, this research has provided a new perspective into the workings of fruit photosynthesis and how it operates under its distinct anatomical and metabolic constraints.

My experimental results revealed numerous unexpected and counterintuitive findings. In leaves, SBPase expression positively correlates with photosynthetic efficiency and biomass accumulation (Ding et al., 2016). However, this study showed that this correlation was not present in fruit, as SBPase overexpression failed to improve fruit metabolic traits and instead led to a reduction in photochemical efficiency. The RNAi-SBPase lines demonstrated improvements in sugar accumulation and enhanced photochemical performance under certain light intensities at ambient temperatures. Essentially, the results strongly suggest that in fruit, SBPase is not essential. However, the chilling experiment allows for the argument that SBPase's function in fruit is to aid in suboptimal environmental conditions, such as during cold temperatures. This study provides the insight that increasing the abundance of SBPase in fruit does not impact the nutritional quality in terms of sugar accumulation or carotenoid concentration. However, this result is nuanced, as I have shown that its overexpression likely positively impacts biomass. This result is further nuanced by the fact that silencing SBPase in fruit has no effect on carotenoid concentration but positively impacts sugar content without compromising biomass.

The overexpression of FBPA in fruit produced metabolic but not photochemical changes. Fruit biomass increased while soluble sugar concentration decreased; this indicates that in this unique metabolic environment, FBPA is involved in carbon allocation or possibly water content. Despite this metabolic shift, chlorophyll fluorescence parameters remained unaltered,

showing that carbon partitioning could be modified independently of photosynthetic performance.

These results allow me to reject my initial hypothesis: the rate of CBB cycle turnover can be increased or decreased by differentially expressing essential CBB cycle genes that bottleneck RuBP regeneration. I hypothesised that differential expression of these genes would affect photosynthetic rate and capacity in a manner previously observed in leaf studies; this proved to be an incorrect assumption.

Regarding the SBPase and FBPA lines, it can be strongly argued that there was sufficient overexpression overall to have a meaningful impact on physiology. This can be said as, on average, the SPBase lines had a 2.8-fold increase, and FBPA had a 5.4-fold increase relative to wild type. This is widely accepted to be sufficient for accurate conclusions to be drawn, as ~2-fold change is accepted as the preferred minimum (Munkácsy, Herman and Győrffy, 2019; St. Laurent *et al.*, 2013). Despite lines AW3-30 and AW3-40 achieving fold changes of 2.1, biologically meaningful insights can still be drawn.

Lastly, the knockdown of CAO provided a targeted rebalancing of chlorophyll metabolism, which reduced sugar accumulation and shifted the concentration of carotenoids. Thus, this confirmed that pigment composition directly influences both photochemistry and downstream metabolism in fruit tissues. However, the hypothesised significant decrease in NPQ, increase in PSII efficiency, and thus increased fruit biomass were not observed.

Additionally, no genetic manipulation in this study resulted in an altered rate of fruit development, allowing me to reject the hypothesis. This suggests that the rate of fruit growth and maturation is not directly controlled by the CBB cycle or chl metabolism, and implies that while both pathways are unlikely to be primary regulators of developmental timing. Fruit development is more likely dictated by broader developmental and hormonal signalling pathways involving auxin, ethylene, and gibberellins. These hormones will determine the rate of both growth and ripening, largely independently of local photosynthetic capacity (Fenn and Giovannoni, 2021; He and Yamamuro, 2022; Tipu and Sherif, 2024).

It was assumed that the fruit CBB cycle functions identically to that in leaves; however, this was not completely accurate, primarily due to the nature of fruit morphology. Tomato fruit lacks stomata; what few stomata are present are likely to be ineffective due to the waxy cuticle

and the absence of a spongy mesophyll (Simkin *et al.*, 2020; Ramesh, Paul and Pandey, 2021). Thus, tomato fruit chloroplasts likely receive the majority of the CO_2 from internal sources, not from the atmosphere (Doddrell *et al.*, 2023; Garrido *et al.*, 2023). The predominant supply likely originates from respiratory CO_2 released by mitochondrial metabolism within distal tissues (such as roots, stems, and leaves), which is then reassimilated by the fruit chloroplasts (Simkin *et al.*, 2020; Garrido *et al.*, 2023). This CO_2 is imported via the pedicle and wider vascular system (Bloemen *et al.*, 2013; Gessler, 2017). This reliance on respired CO_2 results in the fruit CBB cycle functioning more as a recycling mechanism that refixes metabolic CO_2 (Berghuijs *et al.*, 2017; Sui *et al.*, 2017). Thus, carbon availability may be the primary limiting factor of fruit photosynthesis. This provides a reasonable explanation as to why my bids to improve fruit photosynthesis are dissimilar to the results observed in leaf studies.

Taken together, these studies demonstrate that fruit photosynthesis is not as simple as a scaled-down version of leaf photosynthesis; it is regulated by unique anatomical, biochemical, and developmental limitations. However, I demonstrate that modifying fruit photosynthesis and/or photosynthetic apparatus can be a viable method to alter biomass and sugar content, but not the rate of development. Thus, I successfully fulfilled my primary aim to determine the contribution of tomato fruit photosynthesis towards development and nutritional quality with the use of genetic manipulation.

Overall, the findings of this thesis underline the importance of treating fruit photosynthesis as an independent regulatory system when designing methods to improve crop yield and quality. Additionally, this study continues to highlight that improving fruit photosynthesis is a worthwhile method to achieve the full potential of crops. I identify that the strategy by which this will be achieved must be carefully planned, as NFP is a unique process with often nuanced results, specifically when altering the CBB cycle and the fruit's ability to capture PAR.

6.7 Agricultural Relevance and Potential Industry Impact

Beyond the mechanistic significance of the results, this study can have important implications for agriculture and the food industry. The market value of tomatoes is strongly influenced by

sweetness, flavour, colour, and nutritional content. The enhancement of these traits via manipulation of fruit photosynthesis can be a powerful method to improve consumer appeal, satisfaction, and nutritional value.

My most promising finding from an industrial perspective is the increase in soluble sugars observed in iSBPase fruit. This is because sweetness is a major basis of consumer preference and market competitiveness, and the ability to enhance Brix without yield penalties presents a clear biotechnological opportunity. As sugar accumulation is related to flavour and postharvest quality, these results could be used to directly advise breeding strategies aimed at premium tomato cultivars with enhanced market value.

The metabolic shifts observed in iCAO fruit also carry potential significance. Carotenoids such as lycopene and β-carotene are valued for their health-promoting properties, and the ability to alter their proportions through targeted manipulation of pigment metabolism could support the development of tomato varieties with a customised nutritional profile. However, the reductions in sugar content within these lines represent an undesirable trade-off. This trade-off may be solved through iCAO being expressed in tandem with other genes to boost sugar concentration. My demonstration that carotenoid composition can be engineered through fruit-localised chlorophyll metabolism provides a foundation for future research into nutritional enhancement.

From a commercial standpoint, fruit FBPA overexpression may not have particularly immediate benefits. Nonetheless, the results demonstrate that metabolic partitioning can be adjusted in fruit. In addition, it must be noted that while reduced Brix is not commercially desirable, the increase in fresh biomass suggests potential utility in breeding programmes aimed at processing tomatoes, where fruit size is prioritised. More broadly, the demonstration that CBB enzyme manipulation can shift metabolic allocation without altering photochemistry provides new insight into the flexibility of sink metabolism.

These findings are not limited to tomato, as numerous fleshy fruit crops, including peppers, cucumbers, and grapes, possess photosynthetically active pericarp tissues that contribute to sugar accumulation and carotenoid concentration. The principles established here may therefore have a wider relevance across horticulture, by providing a framework for fruit in the future, across a diverse array of species, all containing engineered photosynthesis.

In conclusion, this study has the potential to aid in the tailoring of sugar content, pigment composition, and biomass in future fruit crops. This can help the industry meet consumer demand for improved flavour and nutritional quality, while also enhancing market competitiveness for growers.

6.8 Future Directions

The results of this thesis strongly suggest that single-gene methods are insufficient to produce major gains in fruit photosynthesis. Future work should include a more integrated approach by combining modifications of multiple CBB cycle enzymes with additional strategies that can help improve the inherent anatomical and biophysical limitations of fruit. The enhancement of internal CO_2 availability through altered cuticular properties or stomatal density and strengthening sink strength through enhanced sugar transport and phloem unloading are all promising avenues that could be explored alongside CBB cycle differential expression.

The lack of stomata present on tomato fruit resulted in my reliance on chlorophyll fluorescence imaging over gas exchange measurements, as I deemed gas exchange measurements to be largely unfeasible for the scope of this study. The observation of tomato fruit gas exchange would have required the creation, optimisation and implementation of custom equipment. From a mechanistic viewpoint, the downsides of using chlorophyll fluorescence imaging over gas exchange include: gas exchange measurements can provide the absolute and quantitative rates of assimilation (μmol CO_2 m^{-2} s^{-1}) (Busch *et al.*, 2024), whereas fluorescence imaging provides relative efficiencies (Lichtenthaler and Miehé, 1997; Murchie and Lawson, 2013). In other words, chlorophyll fluorescence imaging provides an indirect proxy for photosynthetic efficiency that reflects how absorbed light energy is partitioned between photochemistry, heat dissipation, and fluorescence. Despite this, chlorophyll fluorescence imaging is a widely accepted, established and reliable proxy for photosynthesis and was a highly suitable method for my purposes (Murchie and Lawson, 2013). Further studies could utilise a custom, highly sensitive gas exchange measuring device to supplement my characterisation of the effects of the gene manipulations.

Additionally, omics-based analysis that joins transcriptomic and metabolomic methods could provide a holistic understanding of how fruit photosynthesis interacts with carbon allocation across the whole plant. This method could allow for the identification of the specific regulatory bottlenecks within this unique photosynthetic system, and thus, future research can be better designed to target the pathways or processes that will have a more predictable impact on fruit. Furthermore, future studies should assess how modifications to fruit photosynthesis will interact with abiotic factors such as light intensity and temperature, in addition to plant age, as these factors strongly influence photosynthetic performance and fruit quality in industrial settings.

Additional research should explore the feasibility of using other genome editing technologies, such as CRISPR/Cas, to introduce the precise modifications to NFP. This particular method could fast-track the development of new cultivars containing improved traits while largely avoiding the challenges related to regulation and consumer acceptance surrounding transgenic crops/produce.

6.9 Bibliography

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