EARLY FRUIT DEVELOPMENT IN APPLE: ANALYSES OF FRUIT PHOTOSYNTHESIS, RELATIVE GROWTH RATES, AND INTERNAL OXYGEN CONCENTRATIONS

by

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(Under the Direction of Anish Malladi)

ABSTRACT

Fruit development in apple includes phases of cell division, cell expansion, maturation, and ripening that can be chronologically divided into early, middle, and late fruit growth. Early fruit development is largely mediated by rapid cell division, while growth at later stages is achieved through cell expansion. Resource availability, intrinsic capacity for growth, and internal atmosphere are major factors that influence each individual plant's growth, particularly during early stages of fruit development. Fruit growth is substantially reliant on carbohydrates transported from nearby leaves *via* the vascular tissues, into the fruit. However, non-foliar photosynthesis is commonly noted in organs such as stems, petioles, petals, sepals, seeds, and fruits. The capacity for fruit photosynthesis and its contribution to early fruit growth are not well understood in apple. In this study, fruit-shading was used to understand the contribution of fruit photosynthesis to early apple fruit growth. Fruit tissues, particularly the peel and cortex, display chloroplast ultrastructure and chlorophyll pigment characteristics consistent with capacity for fruit photosynthesis. Shading resulted in enhanced degradation of plastid components, particularly in the pith. The decrease in fruit photosynthesis by shading, however, did not reduce

early fruit growth or fruit retention. It did affect fruit metabolism, resulting in reduced consumption of carbon resources as indicated by metabolite and transcriptome analysis. Transcriptome analyses also indicated enhanced stress responses upon fruit shading.

Relative growth rates (RGR) were used to perform transcriptomic analyses of fruits with differing growth rates. During early development (0-30 days after full bloom), fruit with high RGR were associated with enhanced cell production as indicated by sustained expression of multiple cell division-related genes. Early fruit development in apple was also associated with chronic hypoxia development, which was associated with high rates of respiration. While chronic hypoxia was partially alleviated by a decrease in fruit respiration at later developmental stages, this was not consistent across cultivars, indicating that other factors such as tissue pore space development may also influence hypoxia development during apple fruit development.

Together, data from these studies indicate that fruit photosynthesis may be dispensable for early fruit growth but alters fruit metabolism and stress response in apple. Further, early fruit development is associated with intensive cell production, the extent of which can influence fruit RGR. Additionally, chronic hypoxia develops in internal tissues during early fruit development, potentially owing to the high respiration rates observed during this period.

INDEX WORDS: *Malus x domestica*, fruit photosynthesis, RGR, hypoxia

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DEDICATION

I would like to dedicate my academic journey to the younger version of myself. We did it!

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To my mother and sisters. For over a decade you all have helped me manage stressful times away from home and given me reasons to look forward to the future.

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LITERATURE REVIEW

INTRODUCTION

Angiosperms spend entire life cycles with the goal of making it to a reproductive phase to produce fruit. Plants and fruits have evolved to be expertly adapted to different environments and can exhibit a wide range of traits that help identify and categorize them based on physiological properties. For example, fruits can be fleshy, dry, dehiscent, indehiscent, aggregate, or simple, and can exhibit different metabolic properties such as being climacteric or non-climacteric. The regulation of fruit growth requires the rigorous and synchronized effort of many processes functioning in sequence. A major such process is photosynthesis. Photosynthesis in all cases is the primary supplier of carbohydrates to the fruit and provides the necessary sugars to be used for respiration and growth. Leaf photosynthesis is the primary but not the only type of photosynthesis to occur in angiosperms. Among other structures, there is evidence suggesting that stems, seeds, and fruits can photosynthesize as well (Aschan & Pfanz, 2003; Simkin et al., 2020). For example, tomato (*Solanum lycopersicum*) fruit display 41% of the electron transport chain capacity of leaves (Piechulla et al., 1987). The contributions of fruit photosynthesis are important to understand as they have implications for the final yield of plants and could possibly be altered to make more productive plants.

Photosynthesis occurring in internal fruit tissues would also have implications for the internal conditions of the fruit. Leaves are laminar organs, allowing for excellent diffusion of

gases to perform photosynthesis due to their ability to readily access resources. However, fruits are often thicker, three-dimensional organs and have different physical properties in comparison to leaves, which can make gas diffusion and photosynthesis more difficult. Thus, the possibility for fruits to contribute to their own growth through photosynthesis is usually restricted to the early phases of fruit growth (Garrido et al., 2023).

FRUIT GROWTH AND DEVELOPMENT

Apple is among the most well-studied plants in the *Rosaceae* family and is one of the most popular temperate fruits worldwide. Globally, fresh apples were anticipated to be produced in excess of 83.7 million tons in 2024, but they are also very valuable to the economy of the United States and make up a large portion of fresh fruit sales (Foreign Agricultural Service, 2024). In the United States, apples are one of the most easily-recognizable fruits in the grocery stores and are significant to the culture of the United States, as apples have been part of American life since pilgrims came to North America to establish a new country (Volk & Henk, 2016). Profitable fresh fruit production relies on harvesting apples of a desirable size, shape, color, texture, soluble solids, and several other traits that are influenced by environmental, management, and genetic factors (Musacchi & Serra, 2018). Thus, understanding all facets of the growth and development or fruit is critical to the success of apple fruit production.

Fruit growth and development are complex processes that require extended and concerted action of phytohormones, appropriate environmental conditions, and proper genetic induction to be adequately executed. In addition to the hormonal and genetic regulation of all stages of growth, various other forms of monitoring such as reactive oxygen species detection

and sugar signaling are also implicated in controlling aspects of growth. The monitoring of the fluctuations of sugars is known to influence nitrogen uptake, secondary metabolism, photosynthesis, and hormone balances themselves (Durán-Soria et al., 2020). Overall, fruit development involves several stages that begin with at pollination and fruit set and ends at maturation and ripening. Major phases of fruit growth and development involve key stages that mark significant changes in the transcriptomic profile of genes to induce changes in response to environmental signals. Typical stages include fruit set, early fruit development mediated by cell division, mid-fruit development mediated by cell expansion, maturation, ripening and senescence (Tijero et al., 2021).

Pollination is the process of pollen grains successfully being transferred onto stigma after being moved from the male anther to the female reproductive pistil. Subsequently, the pollen tube elongates until it reaches the ovary, and a process called double fertilization occurs whereby one sperm cell will fertilize the egg cell to form the embryo and the other sperm cell fuses with the remaining cell in the ovule to create what will become the endosperm (Jahed & Hirst, 2023). Fruit set is the stage of fruit development right before cell proliferation, and is known to be highly regulated by hormones such as auxin, gibberellins (GAs), abscisic acid (ABA) cytokinins, and ethylene, some of which can induce parthenocarpy when applied externally (An et al., 2020; Fenn & Giovannoni, 2021). These hormones are critical in regulating the differentiation of floral organs into fruit morphogenesis as floral organs quickly senesce and fruiting organs are rapidly established like central replum, false septum, and valves in tomatoes (Seymour et al., 2013). The fruiting organ differentiation in apple is unlike those in other fruits as they are classified as “false” fruits. The edible cortex is not the true ovary as in most other

fruits and is instead the hypanthium (Ireland et al., 2013; Xiang et al., 2016). The hypanthium is the fused basal portions of sepal sand petals and is often referred to as the floral cup (Marondedze & Thomas, 2012). The inedible "core" contains the proper ovarian tissue and represents a "true" fruit. Because of this distinction in the origin of fruit tissues, the overall growth of apple fruit may have characteristic differences in growth to many other fruits.

After anthesis of flowers, pollination, fertilization, and fruit set, many fruits undergo a period of growth facilitated by a high rate of cell division. The length of this period differs across species, with tomatoes having a period of 7-10 days of cell proliferation and apples having a period of approximately 5 weeks (Gillaspy, Ben-David, and Gruissem' 1993.; Nosarszewski et al. 2004). Some of the proteins needed to facilitate this division are related to proper spindle assembly, cyclins, cyclin dependent kinases, and tubulin (Livanos, Apostolakos, and Galatis 2012). After the phase of cell division, growth of the fruit is mediated by post-mitotic cell expansion, which can continue fairly late into fruit development depending on the species. In apple, the cell division phase is the most critical for final fruit size, as it determines the final number of cells which can undergo subsequent expansion (Harada et al., 2005).

In many fruits, this middle phase of fruit development is when much of the starch in the fruit is synthesized and stored. Over time, starch will be degraded and the resulting sugars contribute to the soluble solids content of the fruit and comprises sugars and other molecules such as acids (Thammawong & Arakawa, 2007). Soluble solids content of apples is one of the major factors in determining whether fruits are ready for market (L.-Y. Zhang et al., 2004). Apples and other several plants of the *Rosaceae* family are examples where the main translocating sugar is sorbitol instead of sucrose, and the interconversion of sorbitol and

sucrose makes expression patterns of metabolic genes different from other fruits (M. Li et al., 2018). Further, fructose and glucose are often the most abundant sugars even though sorbitol is the main translocated sugar, demonstrating the substantial metabolic conversions of carbohydrates (Loescher et al., 1982) .

Maturation and ripening bring about another significant shift in the transcriptomic profile and metabolic program of fruits. In fruit such as oranges, the entire genetic methylation profile is altered as well, to repress genes such as those involved in photosynthesis, and to activate those associated with abscission and ripening (Huang et al., 2019). Proteins such as expansins are upregulated to contribute to cell wall softening (Dheilly et al., 2016). Many volatiles, organic acids, and sugars are produced to increase the attractiveness of the fruit to herbivores, and the fruit generally becomes more flavorful than at earlier stages of fruit development (Aprea et al., 2017). Visual changes also occur, with the peel of fruits often changing in color and increase in carotenoids or anthocyanins making the fruit visually appealing for humans and other dispersal agents. In apple, transcription factors such as *MYB* increase in abundance to enable greater anthocyanin production leading to increased peel pigmentation (Kviklys et al., 2022).

During this time of change, many fruits exhibit features associated with being climacteric or non-climacteric. Climacteric fruits display a sharp increase in the rate of respiration and production of ethylene, whereas non-climacteric fruits do not often display such changes (Fenn & Giovannoni, 2021). Ethylene is a gaseous hormone generally associated with ripening and senescence, and the production of ethylene is often monitored in postharvest storage to prevent or encourage ripening in fruits like in apples or bananas (Liu et

al., 2020). Many fruits, like apples, tomatoes, bananas, and avocadoes, are climacteric, whereas oranges or strawberries are non-climacteric. However, the categorization of climacteric or non-climacteric is not binary, with many fruits like blueberry falling somewhere in between, representing a spectrum more than a simple categorical bin in which to be placed (Paul et al., 2012; Y.-W. Wang et al., 2022). Ethylene is generally processed in two systems. In System I, the production of ethylene is inhibited by the presence of ethylene, as in early fruit development where excess ethylene is strictly regulated. However, because apples are climacteric, the increase in ethylene is associated with System II ethylene production, where the production of the hormone is auto-catalytic and encourages more ethylene to be produced. Ethylene is a master regulator of ripening, with the degradation of starch being very sensitive to ethylene in apple (Doerflinger et al., 2015). During storage or before harvest, 1-methylcyclopropene (1-MCP) treatment of apples reduced the rate of ripening (Sun et al., 2021). The chemical, 1-MCP is an ethylene inhibitor and binds to receptors to inactivate them and prevent physiological responses of apple to the hormone (Watkins, 2006). As a part of the controlled environment storage methods of apple, storage warehouses also have cool temperatures, higher CO_2 concentrations, and low concentration of O_2 in the atmosphere to discourage respiration. This combination of a high CO_2 and low O_2 atmosphere further prevents ripening of the fruit (Brizzolara et al., 2019; Weber et al., 2015).

After stages of ripening, fruit senescence is noted, where fruits are susceptible to disease and injury from very soft cell walls due to redistribution of resources such as calcium (Hocking et al., 2016). Even with non-climacteric fruit like cucumber, exposure to ethylene accelerates electrolyte leakage, watersoaking, and decaying of fruit as the production of

ethylene is closely related to storage shelf life and decomposition (Paul et al., 2012). While the stages of photosynthesis and cellular division, cell expansion, starch accumulation/decline, sugar metabolism, maturation, and ripening occur in almost all indehiscent fleshy fruits, these timepoints for apple specifically peak around 0-30 days after full bloom (DAFB) for photosynthesis and cellular division, approximately 100 DAFB for cell expansion and sugar metabolism, 90-100 DAFB for starch accumulation, 100-110 DAFB for starch decline, 80-140 DAFB for maturation, and ripening generally extends from 150 DAFB onwards as ethylene rises (Tijero et al., 2021).

EARLY FRUIT GROWTH AND DEVELOPMENT

The period of 0-30 days for early apple fruit growth is critical to the final fruit size due to the establishment of the number of cells that will eventually expand. It has long been reported that the difference in fruit size for apples is rooted in the number of cells that are produced in the beginning stages of growth, with larger fruit having up to 4 times the number of cells as small fruit (Bain and Robertson 1951). The length of time that the cell division period lasts various by species with some stages lasting for a month, 10 days, or up until harvest in the case of plums, sour cherries, and avocados, respectively (Patricia Denne, 1963) . Because apples are composed of tissues that are not truly ovarian, they have different growth properties especially during early fruit development stages. The fused hypanthium that makes up the cortex grows at a rate five times faster than the pith during the first 30 days of fruit growth (Jing & Malladi, 2020). This extensive growth is mediated by an upregulation of cell-cycle related genes such as *AINTEGUMENTA* genes *MdANT1* and *MdANT2*, *B-TYPE CYCLIN DEPENDENT KINASES (CDKs), A*

and B type cyclins, a *WEE* kinase, and an E2F transcription factor *MdDEL1* that were all found to be positively correlated with early fruit growth (Malladi & Johnson, 2011). Genes such as these are easily among the most important for cell division, with many controlling cell cycle progression (Inagaki & Umeda, 2011). A total of 814 differentially expressed genes in apple were found to be specifically associated with early fruit growth in relation to cell division between 14 to 35 days after anthesis (Janssen et al., 2008). Cell proliferation consists of the cell going through many rounds of mitotic cell division. In the plant cell cycle, there are the phases of G1, S, G2, and M (Qu et al., 2021). Across these phases, the cell prepares for cell growth, synthesizes new copies of DNA, performs any genetic repairs, and then goes through the process of cytokinesis, respectively (Dewitte & Murray, 2003). Cyclin dependent kinases are critical in the progression of the cycle by phosphorylating essential proteins that are needed for the cell to continue onto the next phase of division (Tank & Thaker, 2011). In addition to cell proliferation and cell expansion, some fruits also display endoreduplication-associated increase in cell size affecting fruit growth (Harada et al., 2005). Although there has been one instance of endoreduplication in apple, where fruit cells had a ploidy of 4C in comparison to the 2C ploidy of leaves, this is not common (Malladi & Hirst, 2010).

While cell division and cell expansion are the major hallmark processes occurring during early fruit development, the physical fruit is physiologically much different than later stages of growth. Young fruit possess active stomata and do not have the thicker cuticles that later stage fruit do, which has big implications for gas exchange and associated processes like sugar signaling that will eventually lessen over time (Lugassi et al., 2020). There is also a higher concentration of chlorophyll in younger tissues than in later stages, as the degradation of

chlorophyll is often an indicator of ripeness (Seifert et al., 2014). Water relations within the fruit also have dynamic roles in all stages of fruit development. Even in later stages of maturity and in postharvest storage, the phrases “mass loss” are synonymous with “moisture loss,” as most of the fruit mass is lost through transpiration which negatively impacts the marketability of fruit (Lufu et al., 2019). In apple, the stomata gradually become inoperable and turn into hardened lenticels (Khanal et al., 2020). Focusing on early fruit development, the functionality of xylem tissues is crucial for water and associated solute delivery to the growing fruit (Drazeta, 2004). During this time, the stomata of apples are still functional, and are a major source of water loss through transpiration. Even in tomatoes, although there are no stomata present, the broken trichomes on the surface of the fruit causes extensive water loss through cracks and scars (Fich et al., 2020). Having functional xylem tissues is critical to replenish the water that is lost through transpiration during the day (Boini et al., 2022). Apples are also susceptible to xylem backflows, along with other fruits like kiwi and grape, when the water potential in the leaves is more negative than the water potential in fruit (Rossi et al., 2022). The peel of the apple at this stage also is not covered in thick cuticular waxes to prevent further water loss, making the vascular connections especially important during early fruit development. Even in grape berries, the water status in the early stages of development affected cell division and elongation, with water stress negatively affecting cell expansion (Hernandez-Montes 2020). The xylem functionality is slowly decreased over time, with dysfunctional vessels occurring because of the crushing forces of the rapidly-expanding apple fruit cortex (Larson et al., 2023). As fruit mature, the functionality of the xylem tissues is lost and the fruit are completely dependent upon phloem for acquiring water and nutrients. Thus, the connections between respiration,

photosynthates from leaves, water loss, and source-sink relationships to early growth stages are both apparent and deeply intertwined.

NON-FOLIAR PHOTOSYNTHESIS

Through the metabolic activity of RuBisCO, leaves specifically evolved to utilize light and carbon dioxide to convert them into usable forms of carbon, as RuBisCO is the primary enzyme capable of using atmospheric CO_2 and assimilating it into organic matter through the Calvin-Benson Cycle (Tabita et al., 2007). RuBisCO can make up 30-50% of total soluble protein within a leaf (Erb & Zarzycki, 2018). It is the most abundant protein on Earth, with four different forms being found in a variety of different photosynthetic organisms including obligate anaerobic archaea which have Form III RuBisCOs (Ślesak and Kruk 2017). RuBisCO is the primary reason why the Earth has oxygen in its atmosphere, with current land plants continuing to interact with the atmosphere in ways to influence increase biomass with the increasing CO_2 (Keeling et al., 2017). With plants evolving and coexisting with such a life-changing adaptation for millions of years, it is not far-fetched to discover that leaves are not the only plant organs performing photosynthesis.

Photosynthesis in non-foliar organs has been exhibited in many non-leaf structures such as seeds, stems, bracts, petals, and fruit (Brazel and Ó'Maoiléidigh 2019). While the process of photosynthesis in leaves continues until senescence, photosynthetic activity in non-foliar structures like fruits are likely restricted to very early phases of fruit development when tissues are small and green. Obvious differences exist in the comparisons between foliar and fruit

photosynthesis such as light interception, water use efficiencies, and the potential rates of photosynthesis.

Regarding light distribution in classic foliar photosynthesis, orchard workers spend much time increasing the light interception of trees so that light is not “wasted” on areas of land that are not productive. High density plantings aid in light interception while different training styles tend to maximize light distribution within a canopy. The amount of light distributed within a canopy is highly varied with most of the outer leaves having the most access to sunlight due to their placement on fruit trees. The amount of light that impinges upon a tree is also increased when rows are planted in a North-South direction, such that the sun is constantly shining upon the orchard trees in a perpendicular fashion. Leaves perfectly evolved to intercept light, with unique adaptations being developed to track or avoid the sun as in the case of paraheliotropic or diaheliotropic leaves and flowers (Serrano et al., 2018). However, even with excellent row orientation and pruning in an orchard, the amount of light distributed to trees can rapidly be reduced to 20-30% that of the outside of the tree as you go inwards towards the center of the canopy (Jakopic et al., 2009). Some papers report the outside of a canopy receiving 43% of the available light, while the inside receives only 12% (Kviklys et al., 2022). Fruits do not have the capabilities to adjust their position to increase or decrease the amount of light interception. Self-shading of fruit by leaves is a problem that all orchards must manage at appropriate times of the year to prevent large economic losses. Management choices such as summer pruning are used in part to increase the amount of light reaching fruit. Previous reports state that light at the pith of apple fruit is approximately only 0.1% of its initial irradiance outside of the canopy, but vascular tissues were shown to have a higher incidence of light interception (Blanke & Lenz,

1989). It is reported that the most internal parts of the apple fruit, such as the pith tissues, receive 0-2% of sunlight, while the peel and more external tissues of the fruit, such as the outer cortex, can receive up to 47% of light (Aschan & Pfanz, 2003). Additionally, the simple fact that apple fruit are very thick also plays a role in the ability of the fruit to adequately intercept light. Leaves are laminar structures that allow for excellent diffusion of light, but fruit do not have the physiology of being so adept at light capture since it must filter through numerous layers of cells compared to leaves. The ability of a fruit to intercept light is already reduced in comparison to leaves, and fruits are also at a general disadvantage during early fruit development when it comes to water use efficiency as well.

Fruit contain plastids like leaves do, although they are generally less abundant as in leaves and often evolve into different form of plastids to better suit the needs of fruits during maturation and ripening. Unlike most fruit, kiwifruit maintain their chloroplasts well into fruit ripening, and research suggests it may be due to differential endogenous cytokinin programs(Pilkington et al., 2013). But in most cases, as in apples, chloroplasts in the peel transition to chromoplasts similarly to the transition that occurs in tomatoes when an increased carotenoid content can be observed synchronously with rapid metabolization and breakdown of chlorophyll products (Egea et al. 2011; Schaeffer et al. 2017). The manipulability of plastids can be observed in chromoplasts as well, since they may be derived from proplastids, chloroplasts or amyloplasts as in the *Arum italicum* berry (Egea et al. 2010). Various forms of plastids include chloroplasts, gerontoplasts, elaioplasts, etioplasts, amyloplasts, and chromoplasts, with each having the dynamic ability to interconvert between at least 1-2 different forms, reversibly (Jarvis & López-Juez, 2013). When in darkness, plastids cannot

complete the typical proplastid-to-chloroplast pathway, because light is needed for chlorophyll biosynthesis(Lindquist et al., 2016). Instead, these plastids become etioplasts, and contain prolamellar bodies that are crystalline arrangements of photosynthetic precursors waiting on detection of light by phytochromes to finish developing (Solymosi & Schoefs, 2010; Waters & Langdale, 2009). Despite these many different types of fruit plastids, they do occasionally share some common features between them. Plastoglobuli are one key component that are as versatile as plastid types themselves. They function in high-light and stress responses and contain neutral lipids such as prenylquinones, carotenoids, and protein that may sometimes be associated with thylakoids when they are present in chloroplasts (Bréhélin & Kessler, 2008). FIBRILLIN was the first plastoglobule protein discovered, and since then it has been revealed that they are integral to the non-photochemical quenching capacity of potatoes and improved the growth of peppers when overexpressed (Simkin et al., 2007). Similar to plastoglobuli, lipid droplets can vary in composition when present in different organs, yet they remain critical in storing triacylglycerols (TAGs) in oil seeds or pollen coats, a feat that vegetative tissues cannot do as well and is important in acting as storage for carbon (Zienkiewicz et al., 2021). Plastoglobuli have also been documented to have high concentrations of plastoquinone-9 (PQ-9), which is normally important in thylakoids during the light reactions of photosynthesis, but is instead theorized to be stored to act as a reserve of singlet oxygen scavengers (Ksas et al., 2018).

Because fruit have such unique physiological properties, the types of photosynthesis that evolve under these unique conditions are also highly varied. Researchers in the past have called for fruit photosynthesis to be defined as an entirely new form of photosynthesis, since it is very

unlike the typical C3, C4, and CAM photosynthesis types found in leaves(Blanke & Lenz, 1989). In Satsuma mandarins, a combination of C3, C4, and shade plant photosynthesis may be performed due to the high number of stomata on the peel (Hiratsuka et al., 2015). It is thought that duplication and neo-functionalization leads to specialized version of genes that allow C4 photosynthesis to be present in wheat ears during grain filling stages even though wheat itself is a C3 grain (Henry et al., 2020). Non-photosynthetic forms of PEP CARBOXYLASE, PYRUVATE ORTHOPHOSPHATE DIKINASE, and NADP-DEPENDENT MALIC ENZYME (PEPC, PPDK, and NADP-ME) are already crucial in providing carbon skeletons while participating in detoxifying and pH-regulating processes as well (Doubnerová & Ryšlavá, 2011). Typically, the type of photosynthesis performed by fruits is independent of that performed by leaves. Most fruits of C3 plants perform a derivative of C4 photosynthesis, but some CAM plants such as pineapple and prickly pear have fruits that display CAM photosynthesis to varying degrees. Researchers observed marked increases in the uptake of carbon dioxide in prickly pear at night, which resembles CAM plants (Nobel & De La Barrera, 2000). Stems are also known to perform photosynthesis in pathways that mimic CAM photosynthesis (Brazel & Ó'Maoiléidigh, 2019). Some fruits will exhibit temporal separations in the occurrence of reactions that mimic CAM photosynthesis, while others will display mechanisms associated with carbon-concentrating mechanisms of C4 plants as in apple (Garrido et al., 2023).The expression of genes relating to PEPC and other C4 enzymes can be attributed to the inability of fruits to acquire sufficient gas exchange under normal growing conditions. In apple, there is sufficient evidence suggesting C4-type photosynthesis even though apple is a C3 plant. The expression of *MALATE DEHYDROGENASE* and *PEPC* enzymes suggest that there is possible refixation of carbon dioxide

as is present in typical C4 pathways. Evidence of the accumulation of malate in the vacuoles of the apple fruit cells during early fruit growth also suggest that apples can photosynthesize (Jing & Malladi, 2020). There has long been a proposed pathway to demonstrate how malate shuttling would function in the context of apple fruit to contribute to metabolic activity and explain the accumulation of malate (Blanke & Lenz, 1989).

Regardless of the type of photosynthesis that is being performed, it is generally unlikely that fruits accumulate a significant portion of carbohydrates from fruit photosynthesis. Fruits are not expected to contribute significantly to the production of carbohydrates, as they are chemically unable to keep up with their leaf counterparts. The exception is that in some cases such as mandarin (*Citrus unshiu*), fruits are able to photosynthesize just as efficiently as shade leaves (Hiratsuka et al., 2015). Because fruit cannot produce the large amount of sugars needed for their growth, they import much of the sugars via the phloem. Increased presence of sugars as a result of fruit photosynthesis could influence the source-sink relationships within the plant, as the process of phloem unloading is driven predominantly by the existing gradient of carbohydrate concentration (Ma et al., 2019). The buildup of sucrose is continually prevented within the fruit via rapid catabolism and storage of the mono saccharide units in the vacuoles, or compartmentation to not interfere with the sucrose gradient (Falchi et al., 2020). Many proteins such as tonoplast sugar transporters (TSTs) in apple fruit heavily mediate the accumulation of sugars in the cytosol and vacuoles and influence other sugar transporters as well (Braun, 2022). The sugars produced by leaf sources and transported by the phloem all contribute to fruit being one of the largest sink organs after shoots.

Currently, the functions of fruit photosynthesis are thought to be to supply energy-rich molecules like ATP and NADPH for growth respiration, provide metabolites, refix CO_2/ O_2, and to aid in the development of seeds (Aschan & Pfanz, 2003; Garrido et al., 2023). Growth respiration occurs when resources are directed to the production of new tissues. The chloroplast and mitochondrion are the sole organelles that produce energy for plants, making photosynthesis crucial for survival and necessary for growth respiration to occur. Extensive retrograde signaling occurs between the chloroplast, mitochondrion, and nucleus as well as crosstalk between the chloroplast and mitochondrion themselves (Crawford et al., 2018; Mielecki et al., 2020). The production of ATP and NADPH also serve as fuel for the synthesis of energetically expensive fatty acids like tocopherols and triacylglycerols (TAGs). Both help to protect the thylakoids in chloroplasts during stressful situations like high-light conditions (Bréhélin & Kessler, 2008; Chapman et al., 2012). Because the processes of chloroplasts and mitochondria are so intricately linked, fruit photosynthesis could provide several functions to both organelles.

The extremely high rates of respiration that occur in early fruit growth would also require the production of metabolites in high amounts. The construction of amino acids begins with metabolites that are produced within the TCA cycle and glycolysis phases of aerobic respiration. Thus, the high respiration rates are fundamentally tied to the high production of secondary metabolites that are also needed to maintain the turgor pressure within cells by acting as osmolytes (Sui et al., 2017). Non-foliar photosynthesis that occurs in the seeds of soybeans and *Brassica napus* is thought to be critical to the synthesis and accumulation of fatty acids in these tissues (Ruuska et al., 2004) . Similar examples can be found in grape berries and

apple where photosynthesis and the presence of plastids are thought to be important to the accumulation of fatty acids or aromatic amino acids (Schaeffer et al. 2017; Breia et al. 2013).

The internal cycling of carbon dioxide and oxygen can be potentially traced back to the endosymbiotic theory (Hohmann-Marriott & Blankenship, 2011). This widely accepted theory suggests that mitochondrion and chloroplasts were both independent and free-living organisms that evolved to rely upon the chemical processes of the other while in the same cell. The products of photosynthesis are need for respiration to occur, and vice versa. Photosynthesis and respiration are metabolically intertwined, and photosynthesis may function to support the processes of aerobic respiration in the mitochondrion during very young fruit growth. During this period of extensive and rapid cell division, there is a very high need for substrates like oxygen to provide the necessary reducing equivalents that occur at the end of the electron transport chain of respiration within the cristae (Raghavendra et al., 1994). The oxygen evolving complex that is located within Photosystem II may very likely be the main producer of oxygen during this time. This specific set of conditions is also due, in part, to the limitations of stomatal presence. Apple fruit generally have between 30x fewer stomata present on the fruit than on leaves (Blanke & Lenz, 1989; Lawson & Milliken, 2023). This severe reduction in pathways that lead to the atmosphere inhibits the diffusion of oxygen and carbon dioxide into the fruit. In cases where high number of stomata are present, as in cucumber fruit, these fruits are often wrapped in plastic in grocery stores because they continue to respire so heavily that they are at risk for water loss(Cazier, 2000). The stomata of apples become fully closed and non-functional over time, which contrasts with the high respiratory capacity of wrapped fruits that continue to have functional stomata. The Photosystem II of fruits is very efficient and comparable to that of

leaves, while some papers report that fruits can refix up to 88% of internal carbon dioxide released as a byproduct of respiration (Sui et al., 2017). Fruits such as tomatoes have also been suggested to photosynthesize to adjust the internal atmosphere of the fruit tissue as well as contribute in small amounts to the polysaccharide concentrations of tomatoes (H.-L. Xu et al., 1997). This is even more likely considering tomatoes do not have any functional stomata at any point in the growth and development of the fruit, and thus rely almost exclusively on the internal fixation of carbon dioxide and oxygen to supply the tissues with the substrates it needs for metabolic processes (Simkin et al., 2020).

While photosynthesis can occur for a wide variety of reasons in fruits, in tomatoes it is thought that photosynthesis contributes significantly to the proper formation of seeds as well. In one study, after tomatoes were engineered to have a reduced amount of GLUTAMATE 1-SEMIALDEHYDE AMINOTRANSFERASE (GSA), a significant delay in the setting of seeds was noted (Lytovchenko et al., 2011). GSA is crucial to the biosynthesis of chlorophyll, and this connection between photosynthetic capability, carbohydrate acquisition, and seed development is also found in the grain filling stages of alfalfa. The pods of the alfalfa seeds were found to have photosynthesized enough to contribute to the filling of the seed grain more than the leaves within the first ten days of podding (W. Zhang et al., 2017). It is reported that seeds are also able to regulate the allocation of nutrients and have an impact on the final fruit shape, as it partially depends on the distribution of seeds within the fruit locules (Jahed & Hirst, 2023). Thus, maintaining the proper growth of seeds is essential to the fruit.

FRUIT GROWTH RATE

Perhaps the best way to truly model and predict growth is through respiration. According to Hansen et al. (2002), while growth may be limited by environmental factors such as nutrient availability, the measurement of respiration is always an equal representation of the growth rate and efficiency of plant growth. However, in trees such as beech, respiration is not exclusively controlled by photosynthesis or the amount of biomass already present, and instead is influenced by a complex combination of non-structural carbohydrates usage and most recent photosynthesis (Collalti et al., 2020). Thus, the processes of respiration and photosynthesis are difficult to parse and ultimately rely on each other. The sugars that are produced via photosynthesis are critical contributors of growth especially owing to the source-sink relationships that are important during early fruit development. The translocation of sugars and the fruit load of the current year even has an impact on the subsequent fruiting for the next year, with some reports on persimmon noting that a lack of carbohydrate reserves causes alternate bearing in following years (Fischer et al., 2013). In other fruiting trees such as walnut, the energy needed to support shoot growth comes from these reserve of non-structural carbohydrates, and the carbohydrate availability for branches may suffer when the tree chooses to redirect some of those carbohydrates to support growth in other areas (Volpe et al., 2008).

With new fruitlets undergoing rapid cell division in the earliest days of fruit development, the energy needed to support this intense proliferation of cells is thought to come primarily from newly-synthesized sugars instead of stored non-structural carbohydrates (Breen et al., 2020). In apple, fruiting spurs can produce either bourse leaves or spur leaves. Spur leaves grow

directly from the fruiting spur, but bourse leaves grow from bourse shoots, which are vegetative growths on the fruiting spur (Elsysy & Hirst, 2017). In the beginning of fruit development, up to 30 days after full bloom, the primary spur leaves supply most of the carbohydrate resources needed to fuel the growth of tissues (Lakso & Goffinet, 2013). After approximately five weeks, the bourse leaves will support the growth of fruit and after the leaves nearest to the fruit are fully grown, extension shoot leaves will then begin to send sugars farther away and the fruits will be supported by more leaves that are farther away from the fruit (Lakso & Goffinet, 2013). This switching of resource providers occurs because fruits are not the only major sink in the beginning of fruit development. As trees begin to grow again, the growth of new shoots is a bigger sink than the growth of fruits, and trees will preferentially send carbohydrates to new shoots instead of new fruits (Beshir et al., 2017; Fanwoua et al., 2014; Lufu et al., 2019). Subsequently, carbohydrates are "freed up" to be sent to developing fruits farther away. This dynamic of having sugars preferentially being allocated at different time to different organs introduces a concept of source-sink relationships that are especially important during early fruit development.

Apples are sink-limited in the beginning growth stages (but, at different stages in the development of the fruit, apples can be either source-limited or sink-limited (Fanwoua et al., 2014). A large portion of what contributes to a fruit's sink strength depends on the amount of respiration, concentration and uptake of sugars, and photosynthesis (Beshir et al., 2017). The strength of the apple fruit to "pull in" sugars in early fruit growth stages is one of the primary reasons that they are limited by the amount of carbohydrates that can be transported to the fruits. In later stages of growth, apple fruits are source-limited, with the tree unable to fully

supply all fruits with the necessary sugars needed to continue growing. This explains why self-thinning or “June drop” occurs, because there are insufficient sugars being made to support every fruit (Archbold, 1992). During early fruit growth, tomato fruits also exhibit sink-limited growth and eventually become source-limited as the growing season extends. This was determined by tracking the sugar and starch content of tomato fruits and stems, as the carbohydrate content is linearly tied to the source/sink balance (T. Li et al., 2015). During times of source-limited growth, plants may exhibit an excess of amino acids. It is reported that free amino acids may be a sign of excess nitrogen availability that the plants cannot use due to a lack of carbon to use along with the amino acids for growth respiration (Burnett et al., 2016).

Apple flower clusters generally have 5-6 flowers in the shape of a corymb, with the centrally-located flower eventually developing into the “king fruit” (Losada & Herrero, 2013). The position of the fruit within the cluster during fruit set and growth contributes to the likeliness of being dropped or the allocation of resources. King fruit, usually have a higher chance of survival and exhibit higher levels of carbohydrates because they bloom first and thus have a slight time advantage over the lateral fruit within the same cluster (Larson et al., 2023). The lateral fruits also develop at different positions and are thus impacted differently by their position in the cluster. Size comparisons of small, medium, and big lateral fruits revealed that small and medium-sized fruits were more likely to fall off when developed on lower positions of the corymb that represents a sort of abscission potential gradient (Botton et al., 2011). However, when hand-thinned to one fruit per cluster, the dominance of the king fruit or higher up lateral fruits was not observed, suggesting that it is not necessarily the position on the cluster that is

important and is instead the competition between fruits in relation to source-sink relationships that will determine the success or failure of fruit growth (Jakopic et al., 2015).

To better understand the genetic underpinnings of what makes some fruit grow faster than others, this significant source-sink relationship must be taken into account when measuring fruits. The physiological differences that influence fruit growth contribute to unequal distributions of resources among fruits. Measuring the growth of fruitlets is generally done by using absolute growth rates (AGR) or relative growth rates (RGR). Absolute growth rate is defined as: $\frac{(D_2-D_1)}{(T_2-T_1)}$ and relative growth rate is defined as: $\frac{(lnD_2-lnD_1)}{(T_2-T_1)}$ where D1 and D2 indicate diameter at times T1 and T2 (Radford, 1967). In contrast to RGR, absolute growth rate (AGR) is a simpler measurement that only calculates the total difference in size from the final and initial measurements. It does not take into consideration that growth itself is not constant. RGR is often a more accurate assessment of the growth rate as it standardizes the growth characteristic measurement based on the initial size of the fruit by using a natural log of the growth characteristic, such that the change in the measured trait is relative to the initial size (Pommerening & Muszta, 2015). AGR can be a more practical way to measure gross change in traits, but it does not consider the difference in developmental timepoints of sampled fruits. RGR does have shortcomings, where the measurements can become less relevant over time when using it for long-lived trees. This concept of plants shifting the biomass allocation over time in a very predictable way is referred to as ontogenic drift (Xie et al., 2012). The partitioning of resources is size-dependent, so it is critical to consider the initial size of fruits when comparing growth rates.

The RGR of fruits can even differ within different tissue types within the same fruit, as in the case of apple where the cortex grows at a faster rate than the pith and constitutes 86-96% of the volume of an apple fruit when pith tissues only make up 4-12% of total volume (Malladi et al., 2020). This is possibly because there is a higher sink strength in the cortex that draws in the necessary metabolites and sugars to support intense growth. Understanding the genetic underpinnings of differential fruit growth rates would be helpful in determining growth mechanisms and developmental regulation. Assuming that fruits within a population will have different rates of growth, observing the differentially expressed genes within fruits that display exceptionally high or low RGR could give an indication of the genes and processes that are critical in facilitating differential growth.

INTERNAL OXYGEN CONCENTRATION

Leaves are composed of palisade mesophyll and spongy mesophyll cell types. The adaxial side of leaves contain the palisade mesophyll cells that are specialized in reflecting light and performing most of the photosynthesis that occurs in leaves due to their perpendicular arrangement with respect to the epidermal layer of cells above them. Below the palisade mesophyll, on the abaxial side of a leaf, the spongy mesophyll cells are generally irregularly-shaped. The inter-cellular spaces in this region function as air channels and passageways for carbon dioxide, oxygen, and water to diffuse into and out of leaves *via* stomatal openings (Borsuk et al., 2022). The internal atmosphere of leaves is extremely variable, with as little as 3% and as much as 73% of the internal volume of a leaf being reserved for air space (Earles et

al., 2018). The presence of CO_2 and O_2 is necessary for both photosynthesis and respiration to occur.

One of the products of the electron transport chain is O_2. Reactive oxygen species (ROS) are produced in only four places within the cell including chloroplasts, mitochondria, peroxisomes, and apoplast and often causes much damage to the integrity of cell walls and cause leakage among other negative side effects (Muñoz & Munné-Bosch, 2018). Combatting the harmful effects of ROS can include options such as non-photochemical quenching under the xanthophyll cycle, the Mehler reaction, also known as the water-water cycle, or ROS scavenging by enzymes such as superoxide dismutase (SOD) (Foyer & Hanke, 2022). Photosynthesis uses CO_2 directly in the Calvin-Benson cycle, also known as the reductive pentose phosphate pathway. The Calvin-Benson cycle occurs in the stroma of chloroplasts using the energy gained from the electron transport chain, or "light" reactions, of photosynthesis. The cycle consists of three main stages with the first stage being carbon fixation followed by reduction, and then regeneration of ribulose-1,5-biphosphate (RuBP) (Johnson, 2016). During the initial carbon fixation step, RuBisCO fixes CO_2 by combining it with RuBP which immediately splits into two molecules of 3-phosphoglceric acid (PGA)(Eaton-Rye et al., 2012). The two 3-C molecules then undergo reduction and become glyceraldehyde-3-phosphate (G3P). One molecule of G3P goes towards the production of sugars, while the other molecules complete the cycle in the last stage of regeneration. Here, RuBP is regenerated so that carbon fixation may occur again. Every gram increase in biomass can be directly tied to the performance of RuBisCO in the Calvin-Benson cycle.

After the Calvin-Benson cycle sends G3Ps to be converted into glucose, that glucose will be used as energy for respiration after glycolysis occurs in the cytosol. During glycolysis, the breakdown of glucose leads to the production of pyruvate molecule that are processed into acetyl CoA and utilized in the tricarboxylic acid (TCA) cycle that occurs in the mitochondrial matrix(Chandel, 2021). The TCA cycle is responsible for the production of energy after the cycling of acetyl CoA into different molecules such as citrate, isocitrate, succinate, fumarate, malate, and oxaloacetate (Araújo et al., 2012). As a result of this cycling, the TCA cycle is the starting point of the production of many of the amino acids. The energy produced from these conversions is then used in the mitochondrial electron transport chain (ETC). The mitochondrial ETC consists of four complexes and a cytochrome C complex that culminates in the production of water after molecular oxygen is used as the final electron acceptor during aerobic respiration (Vanlerberghe et al., 2020). As mentioned, ROS can also be generated from the mitochondrial electron transport chain by leaking from the complexes I, II, and III (Nolfi-Donegan et al., 2020). The complexities of the presence of oxygen within a cell requires a careful balance, as reactive oxygen causes degradation when molecular oxygen is needed for respiration to occur. Therefore, cells have evolved careful mechanisms to detect the presence of oxygen to allow aerobic respiration to continue. In conditions of low or no oxygen, respectively referred to as hypoxia and anoxia, extreme changes in cyclic electron flow and the xanthophyll cycle can occur and shift the metabolic programs of cells (Kyzeridou et al., 2015).

Hypoxia can generally be defined as oxygen levels between 1-5% in plants or below 10% in soils (Loreti & Perata, 2020; Salvatierra et al., 2020). Total anoxia is the condition of a plant or soil being completely devoid of oxygen. Extremely low oxygen is often used for long-term post-

harvest storage of apples. When being stored in controlled environment atmospheres, a combination of low temperatures, low O_2, and high CO_2 prevent excessive respiration from occurring and thus delaying ripening (Cukrov, 2018) . This alteration of the storage environment reroutes the metabolic profiles of fruits and induces many changes in carbon metabolism, such as the use of sucrose synthase (SUS) instead of hexokinase or invertases that consume ATP (Boeckx et al., 2019). Modifications in amino acid synthesis also may induce an accumulation of alanine. During anaerobic respiration, plants will attempt to produce smaller amounts of energy molecules by fermentation of pyruvate into ethanol. However, ethanol easily evaporates and diffuses because it is an alcohol, so converting the pyruvate to alanine allows plants to hold onto more carbon during times of high stress (Bailey-Serres et al., 2012). An increase in alanine accumulation also has implications for γ-aminobutyrate (GABA). The GABA shunt is an important metabolic pathway that breaks down glutamate into GABA and CO2 in the cytosol of cells and responds within 24 hours of shifts in oxygen (Brizzolara et al., 2019; Fait et al., 2008). It is suggested that the GABA shunt serves as a method to obtain succinate during times of stress and that it may also act as a major reserve of carbon storage and an additional means of regulating pH (Araújo et al., 2012).

Accurate sensing of oxygen is thus critical to the balance of physiological processes. The detection of oxygen within fruits is extremely fine-tuned and related to GROUP VII ETHYLENE RESPONSE FACTORS (ERF-VIIs) such as RAP2.2, RAP2.3, and RAP2.12, with RAP2.2 and RAP2.12 appearing to be partially redundant in the ability to activate hypoxic responses in tissues (Bui et al., 2015). These ERF transcription factors are continually being degraded in the presence of O2 as a part of the N-degron pathway (Rachappanavar et al., 2022). In normoxic conditions, the N-

end of the ERFs experience a removal of methionine, which then exposes a cysteine reside that is oxidized by plant cysteine oxidases. This oxidation then leads to arginylation of the ERFs which is then recognized by ubiquitin-protein ligases that tag the transcription factors for degradation (Pucciariello & Perata, 2021). In conditions of low oxygen, there is a reduced occurrence of these oxygen-dependent reactions and the ERFs are stable enough to be transported to the nucleus and induce transcriptomic changes.

Such affects in the gene expression profile include master regulators of signaling like MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs) and SUCROSE NON-FERMENTING KINASE 1 (SnRK1). MAPKs are ubiquitous in eukaryotes and are evolutionarily conserved pathways that are activated in response to abiotic stress (Sinha et al., 2011). Recently, MAPKs were also suggested to have a role in non-abiotic-stress-related events like cell division, with possible targets of MAPKs being cyclins which are necessary as checkpoints along the transitions of the cell cycle (Banerjee et al., 2020). The effects of the migration to the nucleus are increased as SnRK1 is also recruited to induce change. As a serine/threonine kinase, SnRK1 is a master regulator of metabolic function in response to stressors, much like MAPKs. SnRK1 is involved in the constant detection and monitoring of oxygen levels and acts as a meeting point for several metabolic processes under its regulatory control like lipid metabolism, sugar synthesis, and senescence (Kulik et al., 2011).

In normal growing conditions, there have been several reported instances of developmental hypoxia. Some papers suggest that the hypoxic conditions of apple fruit are chronic instead of acute and thus do not represent a source of stress within the plant (Weits et al., 2021). Chronic hypoxia refers to the intentional creation and maintenance of a low-oxygen environment

through currently unknown mechanisms despite plants being in a normoxic environment (Weits et al., 2019). Acute hypoxia refers to situations of sudden removal of access to air that triggers extensive stress responses in the plant. Chronic hypoxic situations may activate pre-programmed cell rearrangement pathways that are not necessarily stressful and are a part of normal developmental changes (Chang-jie et al., 2004). Growth-related hypoxic conditions are regularly required in organs like seeds, galls, anthers, and the meristems of Arabidopsis to function properly. In fact, when genetically modified to have a higher percentage of air, the meristems of Arabidopsis exhibited delayed meristem development. These types of situations will become hypoxic even though the fruits are in normoxic conditions and do not exhibit stress. The long-lasting reduction in oxygen is thought to be necessary for plants in comparison to sudden and acute hypoxia that develops from flooding conditions. Rice plants also adapt to low-oxygen conditions, with reports that rice can even germinate in completely anoxic situations (Magneschi & Perata, 2009). Deep-water rice plants are well-known to be able to survive for months in flooded rice paddies, making them very desirable among rice farmers in some areas of Asia (Loreti et al., 2016). These rice varieties have two methods of surviving these extended waterlogged conditions with approaches that could be described as an "escape" or a "wait it out" strategy. Rice varieties that employ the "escape" strategy have *SNORKEL1* and *SNORKEL2* genes that allow the plant to extensively alter carbohydrate metabolism so that the rice plant may elongate nodes above the waterline at a rate of 25 cm per day and escape the low-oxygen conditions (Zahra et al., 2021). On the other hand, rice varieties that have a "wait it out" approach do so by dramatically slowing their metabolism and are colloquially referred to as SCUBA rice. In either case, exceptionally quick alteration of

carbon metabolism is at the root of the rice plant's ability to survive the extended watery environments. For plants that are not as adept to surviving waterlogging, other strategies exist such as the development of aerial roots or aerenchyma.

One of the coping mechanisms of plants in response to flooded conditions is the formation of aerenchyma. Aerenchyma are large gaseous pores within tissues can have either schizogenous or lysigenous origins. Schizogenous aerenchyma arise from developmentally programming cells to separate under specific developmental conditions, whereas lysigenous aerenchyma form from the sudden stress of a low-oxygen internal atmosphere that causes cells to lyse rapidly (Evans, 2004). Schizogenous aerenchyma are typically more uniform and have a relatively predictable pattern or layout within the context of the tissue, while lysigenous aerenchyma are usually sporadic and lack noticeable organization. However, rice roots have been found to contain non-apoptic lysigenous aerenchyma that develop from being in waterlogged soils (Joshi & Kumar, 2012). Aerenchyma are generally found in roots, as this is the location where the impacts of flooding are the most immediate and severe, but they can be found in other tissues such as fruits in apples and melons (Biais et al., 2010; Habibi et al., 2023). Studies observing hypoxic conditions in maize roots have found that the root cortex continues to respire due to the transportation of oxygen in the aerenchyma alone (Thomson & Greenway, 1991).

When low oxygen occurs and ethylene concentrations increase, the ethylene signal encourages the hypoxic responses that leads to transcriptomic changes in Arabidopsis (Loreti & Striker, 2020). This ethylene buildup in tissues is one of the main causes of aerenchyma as it cannot readily escape when waterlogging of soils occurs or tissues within fruits are too dense to

allow for a low-resistance and easily-accessible pathway to the atmosphere. This sudden buildup of ethylene that is trapped within the fruit or roots is thought to be alleviated by the formation of these void spaces as it allows the gaseous hormone a place to diffuse temporarily. Air voids in the apple fruit and pear fruit are different when observed using micro-CT scans of the internal spaces. These void spaces can take up as much as 30% of the total volume of apple (Herremans, Verboven, Hertog, et al., 2015). In the past, analysis performed with X-ray computed tomography indicated that there is unclear evidence whether the air spaces in apple arise from "extreme schizogeny" or lysigeny (Verboven et al., 2008). More recent reports using X-ray micro CT reveal that the organization of the pore spaces may be more important to the transportation of gases internally (Herremans, Verboven, Verlinden, et al., 2015).

Altogether, fruit photosynthesis is a complex process that is performed for a variety of reasons and affects fruit metabolism in a variety of ways. The investigation of photosynthesis in the early fruit development of apple begins to answer one of the many questions left regarding the contributions of photosynthetic processes during the beginning phases of development.

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CHAPTER 2

FRUIT PHOTOSYNTHESIS

INTRODUCTION

Apple fruit are largely heterotrophic and rely extensively on the import of carbon (C) to maintain energy for growth. Apples typically obtain carbohydrates from either spur leaves or bourse leaves, depending on the developmental stage, as the main contributor to their supply changes over time. During fruit set and early fruit growth, most C resources come from the immediate spur leaves surrounding the fruit. Bourse leaves emerge as the major C suppliers to the fruit from around 30 days after full bloom (DAFB), following a substantial extent of their development. After 30 DAFB, the extension shoot leaves provide much of the carbohydrates needed for fruits (Lakso & Goffinet, 2013). In general, fruit depend on external sources of sugars for almost all of their growth. However, tomatoes can contribute up to 10-15% of their own carbon needs by photosynthesizing and chloroplast-related genes have been the focus of some recent research (Tanaka et al., 1974). Fruit photosynthesis may play an indispensable role in proper seed formation as well, with tomatoes and alfalfa pods using their photosynthates to support development of seeds or for grain filling (Zhang et al. 2017; Lytovchenko et al. 2011). Contributing to the development of seeds seems particularly important to grains and dry fruits such as hickory (*Carya ovata*) and pecan (*Carya illinoinensis*), where fruit photosynthesis contributed more to fruit development in later stages than during early fruit development (Q. Xu et al., 2016). However, fruit photosynthesis has been

documented in many fruits such as grape berries, apples, satsumas, chickpea pods, and cucumbers (Garrido et al., 2023). The unique physiological properties of each fruit such as its thickness, shape, size, and stomatal density influence traits such as gas exchange, light, and water usage. Apples contain many of the required elements for photosynthesis such as photosynthetic pigments, light availability, and stomata, leading some researchers to believe that apple photosynthesis may be more critical for proper seed development (Tijero et al., 2021).

While leaves can perform either strictly C3, C4, or CAM photosynthesis, fruits often display combinations of features from one or more of these types of photosynthesis, and may be considered an entirely new form of photosynthesis (Blanke, 1992). The type of photosynthesis performed by fruit is often independent of the photosynthesis performed by leaves. This may lead to measurable quantities or gene expression of C4 and CAM genes like *PEPC* or *PPDK* in many fruits, as plants that are C3 will have fruits that exhibit C4 characteristics (Henry et al., 2020).

Measuring photosynthetic capacity is typically done using infrared analyzers where the entire fruit or leaf is enclosed in a chamber to measure fluxes of gas or by using chlorophyll fluorescence of PSII, but alternative indirect methods like shading can be used to measure the capacity of photosynthesis as well (Lawson & Milliken, 2023). Shade netting has been used to improve photosynthetic capacity by decreasing the leaf temperature to improve water usage, reduce transpiration, and reduce damage done to PSII caused by excess light (Manja & Aoun, 2019). Shading cloth has been used on apple trees previously to determine its effect on fruit quality, and found that at 20% shading, white or black shade cloths were able to increase fruit

weight (Boini et al., 2022). In other studies using 30% shade cloth on apple trees, researchers found increase concentrations of N, Mg, and S in the shaded leaves which they attributed to increased chlorophyll activity as it was not hindered by photoinhibition (Hirzel et al., 2020b). However, in a similar study by the same group, shaded apple fruits displayed lower K, Mg, and S than shaded leaves did as a result of reduced chlorophyll content and an interaction between the shade cloth over an average of three seasons (Hirzel et al., 2020a). Thus, it may be such that the environmental conditions of a season have an equal or greater impact on fruit development than shading itself. Generally, a shade cloth of 15-30% is commonly used in orchard production, and increasing the shading percentage to 80% induces a reduced level of cell proliferation and expansion of fruits (Dash et al., 2012). Exposure to light also has ramifications for the ability of shoots to export nutrients to fruits, with unshaded shoots being able to export photosynthates 3 weeks after bloom, whereas 70% shaded shoots did not send a comparable amount of photosynthates to fruit until 5 weeks after full bloom (Bastías et al., 2012). This demonstrates the concept of source-sink relationships in trees, and how shaded shoots will preferentially delay sending resources to fruits until other requirements are met first.

During early fruit development, there is strong competition for resources, particularly between shoots and fruits. The distribution of resources is not uniform even within a cluster of fruits, as the king fruit develop earlier than the lateral fruit and therefore emerges as the larger sink (Mert et al., 2007). During this time, it may be important for the apple fruit to meet its C requirements *via* fruit photosynthesis. There is evidence of light distribution in apple being adequate to maintain chloroplast development (Blanke & Notton, 1992). Additional support for apple fruit photosynthesis comes from the accumulation of malate in the vacuoles that can be

seen as an indicator of photosynthesis (Jing & Malladi, 2020; Martinoia & Neuhaus, 2023; Tao et al., 2020). However, the direct contribution of these tissues to photosynthesis and overall development are poorly understood, with most research occurring in mid to later stages of growth. The temporal changes of carbohydrate requirements are not well-documented, as any potential photosynthetic machinery may become nonfunctional over time as fruit become stronger sinks. Additionally, because of the different growth rates of tissues within the fruit, there may even be differences in the source-sink relationships within the fruit. While not primarily performed to contribute to carbohydrate requirements, fruit photosynthesis is thought to be support roles such as internal gas cycling, secondary metabolite production, production of energy molecules, and to support seed development (Galili et al., 2014; Kyzeridou et al., 2015; Lawson & Milliken, 2023; Teixeira et al., 2022). Photosynthesis is therefore a complex process that is performed with the end goal being any number of functions. To suit the varied nature of photosynthesis, plastid morphology, anatomy and physiology must also be varied.

Plastid development in fruit allows for the evolution of many different forms that can interconvert. Starting out as proplastids with undifferentiated features, plastid can evolve into a variety of specialized plastids like amyloplasts, elaioplasts, etioplasts, chloroplasts, or chromoplasts depending on the genetic and environmental cues (Liebers et al., 2017). Generally, apples will have higher concentrations of chloro-amyloplasts as starch begins to build up in the middle phases of fruit growth and the plastids act as storage compartments (Schaeffer et al., 2017). When proplastids do not have access to light, they develop into etioplasts, with crystalline prolamellar bodies. These plastids are photosynthetically inactive as

light levels are too low to completely complete the conversion to chloroplasts, but they are primed to be chloroplasts when light levels are appropriate (Solymosi & Schoefs, 2010). The etioplasts often contain a prolamellar bodies, which are lattice-shaped structures covered in LIGHT-DEPENDENT PROTOCHLOROPHYLLIDE OXIDOREDUCTASE (LPOR) and contain plastoglobuli among other undeveloped structures (Floris & Kühlbrandt, 2021). The number of plastids in fruit cells is species-specific but generally significantly lower than in leaves. Loquat fruit cells contain 20-40 plastids, while fully mature *Arabidopsis* leaf cells can house around 120 plastids each (Lu et al., 2019; Maliga & Bock, 2011). Within fruit plastids there may be arrangements of thylakoids like those of shade leaves.

It is hypothesized that fruit photosynthesis partially contributes to the C requirements of growth during early fruit development. With the existing physiological, genetic, and metabolic evidence of fruit photosynthesis occurring in apples, special attention given to the early phases of fruit development could reveal more information about the contributions of photosynthesis to the growth program of apple fruit during this period. Hence, the main objective of the current study was to investigate the effects of inhibition of fruit photosynthesis on early fruit development. Observing potential changes in transcriptomics, metabolomics, ultrastructural organization, and pigment concentration could potentially highlight the importance of fruit photosynthesis and its contributions to both molecular and whole-fruit physiology.

MATERIALS AND METHODS

Shade study

To investigate the contribution of fruit photosynthesis to the growth and development of early apple fruit, a shading study was performed to specifically inhibit fruit photosynthesis while limiting interference on leaf photosynthesis. A total of 64 'Oregon Spur II Red Delicious' trees located at the Mountain Horticultural Crops Research and Extension Center, North Carolina State University were used for the study. The apple trees were 17 years old and grafted on M111 rootstocks. Trees were trained to a central leader training system and were maintained by the Center staff. The flowering clusters used for sample and data collection were thinned to include only the king fruit and two lateral fruits to encourage competition within the fruit clusters. Treatments included using shade cloth at 30%, 65%, or 80% shade levels and an unshaded control. Four blocks within a randomized complete block design with 16 trees per block was used for the study. These sixteen trees were further divided into four groups, with each group being subjected to one of the four treatments. Each group with four trees together constituted one replicate. For each of the shading treatment, around 1,000 shade bags were prepared using the black shade material. Strips of shade cloth were hot glued together to make a shade bag approximately 5 cm wide and 7.5 cm long in size. To apply our shading treatments to only the fruit, small bags made from the shade cloth were prepared along with a twist tie to secure the bag on the fruit by tying it to the distending branch. Shading was initiated around 16 days after full bloom (DAFB) until they were collected as samples. Full bloom was determined to be the date when approximately 70% of the flowers were open. Each replicate had 250

shade bags that were divided among four trees such that each tree had approximately 62 bagged fruits on the tree.

Fruit samples were collected at 21 DAFB, 28 DAFB, 35 DAFB, and 42 DAFB. During each time point, 20 fruit were harvested. Around 15 fruit were manually separated into peel, cortex, and pith tissues using a razor, frozen in liquid N, and stored at -80° C until further processing. Two fruitlets were sliced and fixed in 2% glutaraldehyde for transmission electron microscopy.

Fruit measurement and retention

All collected fruits had two diameter measurements taken 90° apart on the transverse axis of the fruit using digital calipers. For fruit retention measurements, a total of 29 fruits per treatment per replicate were tagged at the initiation of the experiment. The number of fruit retained throughout the experiment at each collection date was determined.

Chlorophyll extraction and quantification

Chlorophyll extraction was performed on fruit samples using 80% aqueous acetone solution in the dark. The fruit samples were ground using a Qiagen TissueLyser II (Hilden, Germany) under liquid N, and 80% acetone reagent at around 24x of the sample weight was used for extraction. All measurements were normalized to the amount of tissue used for extraction. Ground samples with acetone were vortexed and centrifuged for 5 min at 4 °C. Supernatants were pooled, and 1 mL of the pooled supernatants were loaded into quartz cuvettes and read using a Thermo-Scientific (Waltham, Massachusetts, U.S.) Genesys 10S UV-Vis spectrophotometer at wavelengths of 470, 646, 663, and 710 nm. Two technical replicates

were performed, and the averages of the replicates were used to calculate concentrations of chlorophyll a, chlorophyll b, carotenoids, and total chlorophyll (Lichtenthaler, 1987). The following formulas for the calculations were based on Lichtenthaler et al. (1987):

Chlorophyll a: 12.25 * (663 nm reading) – 2.79 * (646 nm reading)

Chlorophyll b: 21.5 * (646 nm reading) – 5.1 * (663 nm reading)

Total chlorophyll: 7.15 * (663 nm reading) + 18.71 * (646 nm reading)

Carotenoids: $\frac{1000*(470\ nm\ reading) - 1.82*(chl\ a\ total) - 85.02*(chl\ b\ total)}{198}$

Transmission electron microscopy (TEM)

Apple fruit were first sliced on the axial plane so that a radial slice was obtained before being stored in 2% glutaraldehyde. Fruit was dissected into peel, cortex, and pith tissues of approximately 2-3 mm^3 size and placed into glass scintillation vials. The samples were fixed using a 1% osmium tetroxide (OsO4) solution in phosphate buffered saline (PBS) at 4° C for 1 hour before being exposed to a dehydration series using 200 proof ethanol (EtOH). The dehydration series with EtOH began with a solution of 25% EtOH and 75% PBS solution before increasing the EtOH in increments of 25% the volume of the solution up to 75%. After reaching the 75% EtOH: 25% PBS solution dehydration steps increased in smaller increments of 10% EtOH up to 100% EtOH. The samples were then subjected to the resin infiltration steps using Spurr's resin formulation consisting of ERL 4221, DER736, NSA, and DMAE. Once fully infiltrated with 100% Spurr's resin, the samples were polymerized overnight at 70° C in polypropylene mold. Hardened block faces were then trimmed into trapezoids to expose the sample and thin sectioned using an RMC (Tucson, Arizona, U.S.) MTX ultramicrotome to approximately 80 nm in

thickness with a diamond knife. Sections were collected on 200 mesh copper grids and post-stained with 1% potassium permanganate (6-8 minutes), uranyl acetate (30 minutes), and lead citrate (6-8 minutes). Post-stained sections were then visualized on a JEOL JEM 1011 (Japan) at the Georgia Electron Microscopy facility on the University of Georgia campus.

Gas chromatography (GC)

After collecting fruit, the control and 80% shade samples were frozen in liquid N and finely ground into a powder using a Qiagen TissueLyserII (Hilden, Germany). The gas chromatograph used was a GC-2014 Shimadzu (Kyoto, Japan) AOC-20i equipped with a flame ionization detector and an ULTRA-2 5% phenyl-methylpolysiloxane gas-liquid capillary nonpolar column as the stationary phase. The protocol was based on those detailed by Chapman and Horvat (1989,), Jing and Malladi (2020), Acharya et al. (2024), and Yune et al, (2019) with slight modifications. Initial extraction was performed using 0.125 mg phenyl ß-D-glucoside per 1 mL of 100% methanol for each sample, with the phenyl ß-D-glucoside acting as the internal standard. After centrifugation, supernatants were transferred to glass tubes within amber GC vials for N_2 evaporation at 45° C. Oximation was completed by adding 50 µL of a solution containing 20 mg methoxyamine and 1 mL pyridine to each sample. Samples were placed in a heating block at 50° C for 30 minutes before continuing to derivatization steps. Derivatization was done by adding 100 uL of N-methyl-N (trimethylsilyl) trifluorocetamide (MSTFA). Amber vials containing samples were then loaded into the auto-sampler of the GC and processed. Standard curves were developed for each metabolite analyzed, and were used to determine concentrations of the metabolites. Metabolites tested included the following: alanine,

asparagine, citric acid, fructose, glucose, glutamic acid, malic acid, myoinositol, quinic acid, sorbitol, succinic acid, and sucrose.

Starch quantification

An 80% EtOH extraction was performed to remove other metabolites while discarding the supernatant each time after incubating at 80°C for 10 minutes. After incubating with distilled water at 100°C for 10 minutes, an amylglucosidase solution with 35 units enzyme per reaction in 200 mM sodium acetate was used to convert the starch to glucose over a 24 hour period while rotating at 55°C. Glucose concentrations were measured via an enzyme assay containing hexokinase and G6P. Both enzymes were at a concentration of 1 unit per reaction, and the NADH produced from the reaction was measured with a Thermo-Scientific Genesys 10S UV-Vis spectrophotometer at 340 nm before and after adding the enzyme mix (Jing & Malladi, 2020).

RNA Sequencing analysis

RNA sequencing, differential gene expression, and gene ontology analyses were only performed on fruit samples. RNA extraction was completed using a 24:1 chloroform:isoamyl mixture before adding 0.25 volumes of lithium chloride following Jing and Malladi, 2020. Samples were precipitated in a 4° C refrigerator overnight and exposed to 70% EtOH and a 2x SSTE buffer the next day. After another 2 hours precipitating in the SSTE buffer, samples were cleaned with ethanol again and centrifuges. The remaining pellet was dissolved in 25 µL of

DEPC water and stored at -80° C after quality an on analyses using gel electrophoresis and spectrophotometry (Hamburg, Germany).

Illumina paired-end strand-specific eukaryotic mRNA (polyA enrichment) total RNA sequencing was performed by Novogene (China). FastQC analyses were performed using FastQC (version 0.11) to run initial quality control checks on raw reads, and adapters were trimmed using the paired-end option of Trimmomatic version 0.39 (Bolger, A. M., Lohse, M., & Usadel, B. 2014). After adapter trimming, another QC analysis was performed before beginning the STAR alignment. Version 2.7.10b of the STAR alignment program was used to map reads against the GDDH13 genome and gene models downloaded for the same genome reference from the Rosaceae Genome Database (Jung et al. 2019). After all reads were mapped, each sample file generated a "readspergene.out.tab" file which was exported to Excel. The DESeq2 program was then used on the reads per gene output files to determine differentially expressed (DEGs) in the samples. The reference level was set to the control samples, so that the differentially expressed genes are upregulated or downregulated with respect to the Control. The list of differentially expressed genes was used for GO analyses on agriGO v2.0 (Tian et al, 2017). A single enrichment analysis (SEA) for *Malus x domestica* was performed on each subset of high and low DEGs and the reference background genome chosen was the GDDH13v1.1 using Blast2GO.

RESULTS

Fruit diameter and retention

Fruit diameter was significantly different across treatment groups at 21 DAFB (ANOVA; $F(3,308)=2.805$, p-value=0.0399), although Tukey's HSD test did not show significant differences among treatment groups. Fruit diameter was also significantly different 35 DAFB (ANOVA; $F(3,287) = 6.47$, p-value=0.000298). Tukey's HSD analysis revealed that the 65% shaded fruit were significantly larger than fruit in other treatments. At this stage, the 80% shaded fruit displayed the smallest fruit diameter which was not significantly different from that in the control.

Fruit retention was only statistically affected by treatments at 35 DAFB (ANOVA; $F(3,12)=8.66$, p-value=0.00249). The 30% shaded fruit had the most fruit retained over time, with around 88% fruit at 35 DAFB. This contrasted with the control, 65%, and 80% shaded treatments which retained 80, 82, and 75 % fruit by 35 DAFB. However, these three treatments were not significantly different at this stage of fruit development.

Chlorophyll quantification

Higher levels of chlorophyll a and b were present in the peel than in the cortex and pith, and overall trends indicate a reduction in total chlorophyll over time. During early fruit development, peel tissues had increased chlorophyll a fold change of 1.21 and 1.55 in comparison to the cortex and pith, respectively. Similarly, chlorophyll b in the peel was present at increased fold change values of 0.88 and 1.34 with respect to the cortex and pith. Only carotenoids and chlorophyll A displayed any statistically significant changes between the

control and 80% shaded samples from 28 to 35 DAFB. Samples collected at 28 DAFB primarily exhibited differences in the carotenoid concentration. Control and 80% shade samples showed significant differences (p-value < 0.05 for peel and cortex; p-value < 0.01 for pith) in carotenoid levels in the peel, cortex and pith tissues. The concentrations of carotenoids for control samples in the peel, cortex, and pith were 0.0386 $\pm$ 0.00375, 0.0153 $\pm$ 0.000323, and 0.0148 $\pm$ 0.000904 mg/g. The concentrations for 80% samples were 0.0234 $\pm$ 0.00214, 0.0114 $\pm$ 0.00154, and 0.00925 $\pm$ 0.000488 mg/g, respectively. Thus, shade peel, cortex, and pith 28 DAFB displayed Log2fold decreases of -0.722, -0.424,and -0.678 with regard to carotenoids.

Chlorophyll A concentrations were statistically different only in pith tissues during both timepoints (p-value < 0.05 for both). Concentrations for pith 28 DAFB was 0.0235 $\pm$ 0.00183 mg/g for 80% shade and 0.0409 $\pm$ 0.00523 mg/g for control fruits. This translates to shaded fruit having a Log2fold decrease of -0.799. Chlorophyll A concentrations at 35 DAFB was 0.0152 $\pm$ 0.00149 mg/g and 0.0197 $\pm$ 0.00101 mg/g respectively, which translates to a Log2fold decrease of -0.374 . In both the cases of the carotenoids and chlorophyll A, the control samples had higher concentrations of pigment than the 80% shaded fruit.

The remaining chlorophyll B and total chlorophyll measurements were not statistically different between the control and 80% samples at either 28 DAFB or 35 DAFB. To act as a comparison, bourse control leaves were sampled on the 24th of May 42 DAFB and also measured for the same pigments. When comparing chlorophyll a of the control peel, cortex, and pith tissues of 28 DAFB (when pigments were highest), bourse leaves had increased Log2fold values of 2.611, 3.897, and 4.012. In regard to chlorophyll b values were 2.525, 3.419, and 3.496, respectively. Carotenoid values were 2.139, 3.474, and 3.522, respectively. Total

chlorophyll measurements of the bourse leaves had increased values of 2.587, 3.75, and 3.852 for the peel, cortex, and pith.

Gas chromatography mass-spectrometry (GC-MS)

Quantification of metabolites in control and 80% shaded fruit samples indicated that the metabolite composition of the fruit did not generally change in the response to shading. There was not a significant change in metabolites for glutamic acid, citric acid, quinic acid, succinic acid, fructose, glucose, myoinositol, alanine, serine, or asparagine. There were, however, significant changes in carbohydrate distribution in response to the shading of fruit which occurred at different collection dates.

Fruit samples at 21 DAFB displayed statistically significant differences in the fructose concentration between control and 80% shade samples. Control samples contained 10.4 ± 0.719, 14.6 ± 0.943, and 9.69 ± 0.651 mg/g of peel, cortex, and pith tissue. In contrast, the 80% shade peel, cortex, and pith samples contained 9.54 ± 0.904, 16.5 ± 0.617, and 11.3 ± 0.48 mg/g fructose. Other metabolites tested were not statistically significant during this collection date.

The 80% shaded fruit at 28 DAFB exhibited higher sucrose concentrations than in the control fruit. Control peel, cortex, and pith tissues had sucrose concentrations of 1.36 ± 0.191, 1.3 ± 0.118, and 1.22 ± 0.0486 mg/g. In contrast, the 80% shaded fruit had significantly higher peel, cortex, and pith sucrose concentrations of 1.87 ± 0.212, 1.87 ± 0.0994, and 1.67 ± 0.0669 mg/g. The shaded fruit had increased Log2fold values of 0.459, 0.525, and 0.452 for the peel, cortex,

and pith. No other metabolites tested were statistically significant for this sample collection date.

A significantly higher sorbitol concentration was also noted in the 80% shaded fruit at 35 DAFB. The sorbitol concentrations for the peel, cortex, and pith tissues in the control fruits were 2.54 $\pm$ 0.18, 3.52 $\pm$ 0.246, and 5.61$\pm$ 0.345 mg/g, respectively. The concentrations of sorbitol in the peel, cortex, and pith tissues of 80% shaded fruit was 3.31 $\pm$ 0.448, 3.87 $\pm$ 0.0675, and 6.59 $\pm$ 0.345 mg/g, respectively. The shaded fruit had increased Log2fold values of 0.382, 0.137, 0.232 for the peel, cortex, and pith.

Increased fructose, sorbitol, and sucrose accumulation suggests a possible reduction in glycolysis that would prevent the catabolism of carbohydrates and encourage a buildup of sugars. Alternatively, an increased translocation of sugars could explain the increase in carbohydrate accumulation as well. This change in sugar accumulation could be observed as early as 21 DAFB and continued throughout the collection dates.

Starch quantification

The effects of shading on starch concentration were only evident at 35 DAFB. Starch did not significantly differ in the 21 and 28 DAFB samples. At 35 DAFB, the concentration of starch was higher in the 80% shaded fruit across all tissues in comparison to the control. Control samples had starch concentrations of 1.43 $\pm$ 0.13, 1.47 $\pm$ 0.151, and 0.365 $\pm$ 0.0934 for the peel, cortex, and pith, respectively. Samples that were 80% shaded had values of 1.57 $\pm$ 0.278, 2.04 $\pm$ 0.288, and 0.692 $\pm$ 0.0738 for the same tissues.

The largest increase in starch storage was in the shaded pith tissues. Shaded pith tissues contained an additional 89.5% the amount of starch that was found in control tissues. Similarly, shaded cortex tissues accumulated an increase of 38% the amount of starch as in control tissues. Shaded peel tissues accumulated only a slight 9.7% increase in starch compared to the control. Overall, there is strong evidence that shading encourages starch to accumulate within deeper layers of fruit tissue and increase in concentration as you go inwards towards the core of the fruit.

Transmission electron microscopy (TEM)

Analysis of the transmission electron microscopy micrographs revealed temporal and spatial differences in the ultrastructure of the plastids. The peel and cortex tissues generally displayed features consistent with photosynthetic capability. The peel and cortex displayed grana thylakoids and stromal thylakoids and were relatively similar throughout the duration of analysis. The pith often contained hypergrana (extensively stacked membranes). In addition to the hypergranal features, the pith plastids also contained numerous plastoglobuli and osmiophilic bodies. Peel plastids were more numerous, although still at a very small number (generally less than 10), while cells in the cortex and pith generally only had a few (generally 1-4) plastids.

Across the treatments, shaded fruit peel tissues displayed higher occurrence of plastoglobuli. In general, the control and 80% shade fruit samples did not noticeably differ in the cortex. The differences in the control and 80% shaded samples were the most striking within the pith. Shaded pith tissues exhibited a large amount of etioplasts, plastoglobuli, and osmiophilic bodies

in addition to hypergranal stacks. They contained prolamellar bodies within plastids that were not found in the control tissues.

Typical ultrastructural features of plastids at varying stages of development include prolamellar bodies, plastoglobuli, grana, osmiophilic bodies, and starch granules. Temporally, there was a general increase in the presence of starch granules and inclusions in the cortex.

RNA Sequencing analysis

Control and 80% shaded fruit samples collected at 28 DAFB (10 d after initiation of shading treatment) were used in the RNA Sequencing analysis. Differential gene expression analysis performed on peel tissue revealed 370 genes that were differentially expressed between the shade and control fruit. Of the 370 genes, 244 were downregulated genes and 126 were upregulated genes. The DAG tree assembled by AgriGO revealed that the downregulated peel genes carried potentially significant roles in oxidation-reduction processes (p-value = 0.000148) and small molecule metabolic processes (p-value = 0.0445). Some of genes that affected by shading in the peel included *ALCOHOL DEHYDROGENASE* (MD05G1013200), *CYTOCHROME P450* (MD02G1171600), *PEROXIDASE SUPERFAMILY PROTEIN* (MD15G1321200), and *2-OXOGLUTARATE/IRON DEPENDENT OXYGENASE* (MD15G1007000).

The cortex tissue comparisons yielded 37 downregulated DEGs and 263 upregulated DEGs. Of the downregulated and upregulated genes, 278 and 171 were annotated, respectively. The downregulated genes had 2 significant GO terms, and the DAG tree displayed categories of ATP metabolic processes (p-value = 0.0466), carbohydrate catabolic processes (p-value=0.0244), and pyruvate metabolic processes. Some of these genes included *PHOSPHOFRUCTOKINASE*

(MD08G1109700), *FAD/NAD(P)-BINDING OXIOREDUCTASE* (MD15G1064100), and *THIAMINE PYROPHOSPHATE DEPENDENT PYRUVATE DECARBOXYLASE* (MD10G1283500) which decreased in transcript abundance in response to shading.

Comparison of DEGs in the pith tissue indicated a total of 415 DEGs, with 230 downregulated genes and 185 upregulated genes. Within the downregulated genes, the categories that had the most significant p-values showed enrichment of genes associated with carbohydrate catabolic processes (p-value = 0.0022), single-organism carbohydrate metabolic processes (p-value = 0.0022), single organism carbohydrate catabolic processes (p-value = 0.000808), and protein folding (p-value 0.000771). The other processes were of a lower level of significance and consisted of genes associated with nucleoside metabolic processes (p-value = 0.0414), nucleoside triphosphate metabolic processes (0.0458), ribonucleoside metabolic processes (p-value = 0.0361), ADP and ATP metabolic processes (p-values of 0.011 and 0.0414, respectively) and macromolecular complex assembly (p-value = 0.0443) among other categories. Pith tissues displayed reduced carbohydrate metabolism in response to shading with genes such as *PHOSPHOFRUCTOKINASE* (MD08G1109700) and *SUGAR ISOMERASE* (MD08G1034600) displaying significantly fewer TPMs in the shade fruit in comparison to the control.

Among the three tissues there were several overarching pathways or processes that were affected due to shading. These processes included photosynthesis, glycolysis/respiration, hypoxia, and stress responses. There was a downregulation of genes associated with photosynthesis across all tissue types. In the peel, the genes that had > 2-fold decrease in expression due to shading included *RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 1A (*MD09G1252100) and *PHOTOSYSTEM II REACTION CENTER PROTEIN D* (MD09G1235700). In the

cortex, *PHOSPHOENOLPYRUVATE CARBOXYLASE KINASE 1, PYRIDOXINE BIOSYNTHESIS 1.2, RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 1A, THIOREDOXIN F2, PHOTOSYSTEM II BY,* and *PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.2* decreased in abundance due to shading but by less than 2-fold. In the pith, *RUBISCO ACTIVASE* (MD04G1134100) was significantly downregulated, and other genes such as *PYRIDOXINE BIOSYNTHESIS 1.2* and *FLAVONOL SYNTHASE 1* were downregulated as well (Log_2fold changes of -0.641 and -0.498, respectively). Genes involved in glycolysis and respiration such as *PHOSPHOFRUCTOKINASE* were downregulated in all tissues of shaded fruit (MD08G1109700). Additionally, *LACTOGLUTATHIONE LYASE/GLYOXALSE* (MD15G1248200) was also downregulated in the peel.

Interestingly, hypoxia-related genes were not upregulated in the shade in comparison to the control. In fact, in all three tissues, two hypoxia-responsive genes (MD04G1199400 and MD01G1162000) were downregulated in the shade fruits. Additional genes that are markers of hypoxia such as *PHOSPHOFRUCTOKINASE and TPP-DEPENDENT PDC* were downregulated in all three tissues as well. Heat shock proteins were among the most significantly upregulated genes in all three tissues. The 17.6 kDa class II heat shock protein (MD08G1068800) and HSP20-like chaperone (MD17G1209800) were the most significantly upregulated genes across all tissues, with control fruits in some cases having 0 TPMs while shaded fruits had greater than of 450. Additionally, RING/U-box proteins were highly expressed in the pith which is indicative of cell death.

DISCUSSION

Fruit shading does not affect fruit size and retention

Fruit shading was employed in this study as a tool to specifically reduce fruit photosynthesis during early fruit development. Decrease in fruit photosynthesis by shading did not substantially impact fruit growth within the duration of the measurements, even under extreme shading (80%). Further, fruit shading minimally reduced fruit retention within the duration of the experiment. It should be noted that significant competition for resources existed during this period owing to the presence of up to 3 fruit per cluster. Together, these data indicate that lack of fruit photosynthesis did not directly limit growth of the apple fruit during early fruit development. It is likely that foliar photosynthesis, and/or increased translocation of photosynthates into the fruit compensated for reduced fruit photosynthesis.

Fruit peel and cortex tissues display functional photosynthetic components

Fruit contain the necessary pigments, plastidial components, and gene expression required for photosynthesis. Fruit tissues contained significant amounts of chlorophyll and carotenoid pigments during early fruit development, although these levels were up to 6-fold lower than that noted in leaf tissues. Across the fruit, the peel tissue appeared to contain a greater amount of photosynthetic pigments compared to the internal tissues indicating a higher potential for the peel to perform photosynthesis. Reducing fruit photosynthesis by shading reduced accumulation of carotenoids significantly across all tissues by 28 DAFB suggesting an impact of lower light availability on the accumulation of these pigments. Light is known to influence carotenoid biosynthesis in foliar plant tissues (Von Lintig et al., 1997; Zhu et al., 2017).

Hence, reduced light levels and concomitant decline in photosynthetic capacity under fruit shading likely resulted in reduced carotenoid accumulation within apple fruit tissues. Concentration of Chlorophyll a also declined in response to fruit shading, although this occurred specifically within the innermost fleshy tissue, the pith. Consistently, multiple studies have demonstrated that extended shading reduced chlorophyll a accumulation within plant tissues (Zhu et al., 2017; Wu et al., 2021). Together, these data indicate that fruit photosynthetic pigment characteristics are directly affected by decrease in light availability.

All fruit tissues contained chloroplasts although the plastid number per cell was greatest in the peel and lower in internal tissues. Fruit tissues also displayed plastidic ultrastructural features consistent with photosynthetic activity. Specifically, the peel and cortex tissue chloroplast ultrastructure was consistent with photosynthetic activity as witnessed by the presence of normal grana and stroma thylakoids. The pith tissues displayed presence of hypergrana. This was consistent with previous reports of the presence of extensively stacked thylakoids (all-granal thylakoids) in internal apple fruit tissues, and are likely attributable to the constitutive low light intensities encountered within these tissues (Phan, 1970; 1975). Fruit shading generally increased the occurrence of plastoglobuli across the peel and pith tissues. Within the pith, increase in prolamellar bodies, plastoglobuli, and osmiophilic bodies, all of which are associated with associated with the dismantling and recycling of plastids, was noted in response to fruit shading. In most cases these plastidic components are believed to act as storage units for various types of molecules (Eugeni Piller et al., 2012). In stressful conditions where high light causes damage to cellular components, these lipid bodies are thought to behave as reservoirs for antioxidants to help mitigate damage (Espinoza-Corral, Schwenkert,

and Lundquist 2021). Similarly, plastoglobuli and osmiophilic bodies usually develop to act as storage for toxic chlorophyll intermediates such as phytyls and chlorophyllides (Lundquist et al., 2020). To prevent damage to the chloroplast membrane, these compounds must be sequestered into lipid bodies where they are unable to react with membranes (Bréhélin & Kessler, 2008). The increased observance of these various lipid bodies likely indicates restructuring of plastids and potential storage and recycling of thylakoid components. Prolamellar bodies are usually noted in etioplasts when limited light availability prevents complete conversion of proplastids into chloroplasts. When fruits are shaded for extended periods of time and chlorophyll is not needed for plants, etioplasts become the predominant plastid type found within cells (Floris & Kühlbrandt, 2021). Together, the plastid ultrastructural features confirm photosynthetic capacities of fruit tissues such as the peel and cortex. Further, they indicate that fruit shading results in substantial modification of internal plastid ultrastructure to potentially allow for recycling of thylakoid components.

Fruit shading alters C metabolism

Sucrose concentration increased between 28 and 35 DAFB while that of Sor decreased across all tissues, consistent with previous reports (Horikawa et al., 2019; Jing and Malladi, 2020). Further, starch concentration steadily increased across the duration of the experiment in peel and cortex tissues. While concentrations of many of the metabolites measured were generally unaltered, that of three major carbohydrates was significantly altered by fruit shading. Greater accumulation of sucrose was noted at 28 DAFB, while sorbitol and starch concentrations were greater across all tissues at 35 DAFB in the shaded fruit. Considering that

fruit photosynthesis was downregulated in peel by fruit shading as indicated by reduced expression of *PSII REACTION CENTER PROTEIN D* (MD09G1235700; Log_2fold value of 1.16 in control) and *RUBISCO SMALL CHAIN* (MD09G1252100; Log_2fold value of 1.01 in control), increases in Suc, Sor and starch within shaded fruit indicates either enhanced C import into the fruit or a decrease in C catabolism in response to decrease in fruit photosynthesis.

Fruit shading reduces C catabolism, increases storage, and enhances fruit stress response

Overall, it seems that fruit respond to shading through reduced carbon catabolism and adjustments of carbohydrate storage. This can be observed through our RNA sequencing data as well as our GC-MS and starch quantification experiments.

Several genes relating to carbohydrate metabolism were downregulated in shaded fruit. Phosphofructokinase mediates the transition of fructose-6-phosphate into fructose-1,6-diphosphate and functions as the first true step of commitment to glycolysis by catalyzing this irreversible and rate-limiting step in the pathway (H. Wang et al., 2021). Phosphoglycerate mutase (-0.698 and -0.771 Log_2fold change in cortex and pith) also is responsible for catalyzing an essential step in glycolysis (Li et al. 2016). The reduction in gene expression of *SUGAR ISOMERASE* (MD08G1034600; -0.942, -0.824, -1.09 Log_2fold change in peel, cortex, and pith), *PHOSPHOFRUCTOKINASE* (MD08G1109700; -2.205, -1.976, -2.07 Log_2fold change in peel, cortex, and pith), and LACTOGLUTATHIONE LYASE/GLYOXYLASE (MD15G1248200; -4.52 Log_2fold change in peel) indicates the intense shift from a catabolically-intense program to one focusing more on storage. The reduction in all these glycolysis-related genes coincide with the GC-MS results of increased accumulations of sugars in 80% shaded fruits 28 and 35 DAFB. Fruit

are clearly switching to a lower level of catabolism and promoting increased accumulation of sugars. By not breaking down sugars, this accumulation could be interpreted as an attempt for fruits to conserve the carbohydrates they have by also increasing storage. This preservation is observed in the starch quantifications. Starch was significantly different 35 DAFB between the control and 80% shaded in all three fruit tissues, with the largest Log2fold change in starch concentration occurring in the pith. Shaded tissues had increased Log_2 fold values of 0.134, 0.47, and 0.924 for the peel, cortex, and pith. The values increase as tissues become more inward.

In control pith chloroplasts, the hypergrana could be a significant source of reactive oxygen species. The gene expression for PATATIN-like protein (PLP) 9, DNAJ heat shock family protein, chloroplast heat shock protein 70-2, several chaperonins, and several heat shock transcription factors was reduced. PLP is prominent in potato and found to be induced in stressful conditions like bacterial infections and found to be critical in cell death (Gao et al., 2021). It is suggested that PLPs can induce a hypersensitive response in cotton that leads to programmed cell death (Cacas et al., 2009). However, while PLPs are critical in programmed cell death execution, they also are important in lipid biosynthesis pathways to provide resistance to pathogens in the case of *Arabidopsis* against botrytis (Camera et al., 2009). While the pith does not experience much light to begin with, the extended shade conditions could be stressful for the fruit. Heat shock proteins, chaperonins, and PLP are all recruited when high-light scenarios cause damage to photosystems as a result of being overwhelmed.

CONCLUSION

Photosynthesis significantly contributes to the early fruit growth and development of apples primarily through influencing carbohydrate metabolism. The shading treatment altered processes such as photosynthesis, respiration, and sugar accumulation and can be observed through transcriptomic shifts in gene expression. Shading also significantly effects chlorophyll accumulation and plastid ultrastructure. The increased shading is detectable far into the inner tissues of the fruit and alters physiology within the pith despite having little light filter through tissues to begin with. The importance of light and photosynthesis in the early phases of fruit development are thus apparent as the removal of light significantly changes many aspects of growth and development to adjust for a low-light condition. Fruits appear to switch to a metabolic program in favor of carbohydrate conservation by reducing glycolysis and increasing storage of carbohydrates in to starch granules in plastids. However, this did not lead to changes in fruit size.

FIGURES

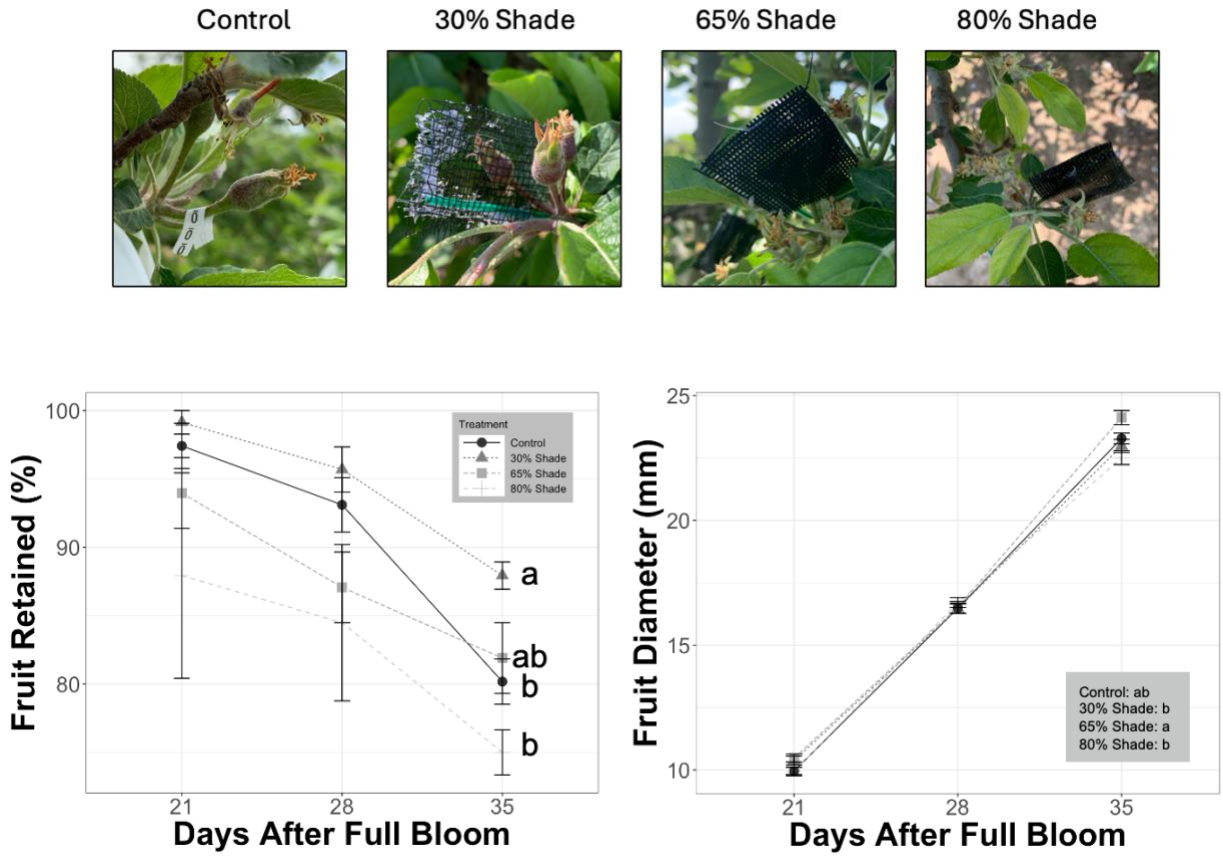

Figure 1. Representative photographs of control and 30%, 65%, and 80% shade treatment bags applied to fruit. Fruit retention and fruit diameter were measured during the early fruit development period in 'Red Delicious' apples. Similar letters next to the treatment symbols indicate no significant difference among treatments within a given date as determined by ANOVA. For fruit diameter data, significance letters are presented within the legend for 35 DAFB, for clarity.

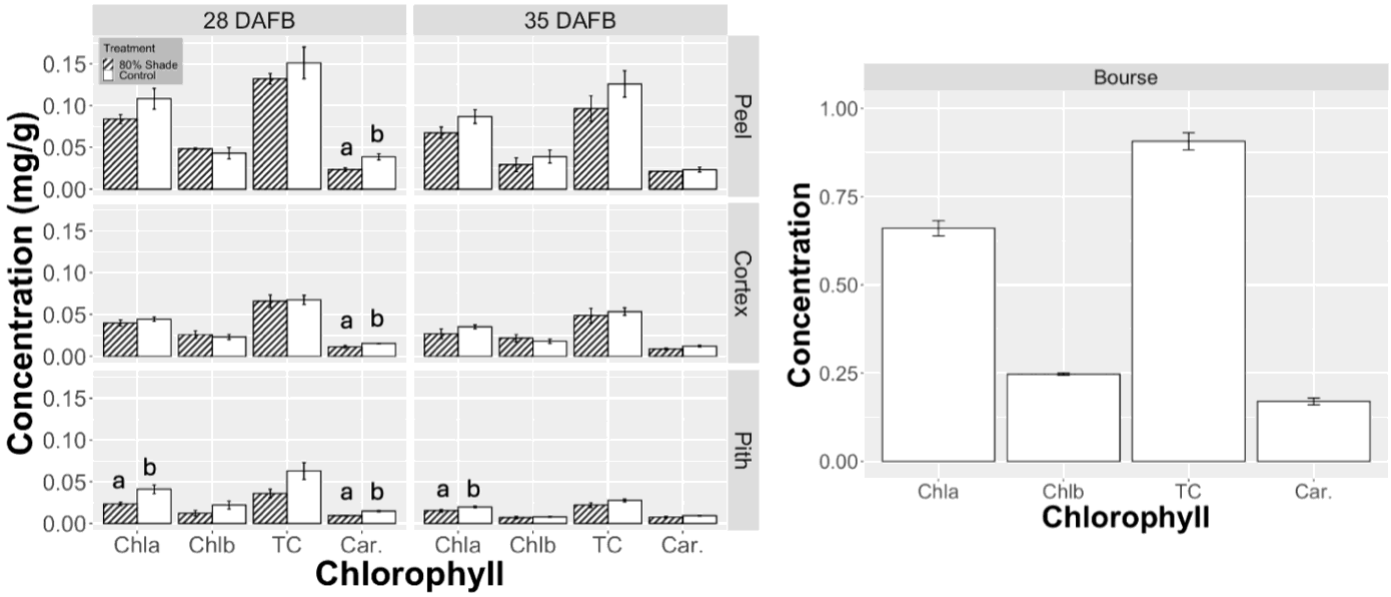

Figure 2. Pigment extractions of chlorophyll a, b, total chlorophyll, and carotenoid. Concentrations were determined during early (28 DAFB) and mid (35 DAFB) fruit development in peel, cortex, and pith tissues. Similar letters above the bars indicate lack of significant differences between treatments. Pigment measurements were also performed for bourse leaves and are presented in the panel on the right, to allow for comparison with fruit tissue pigments.

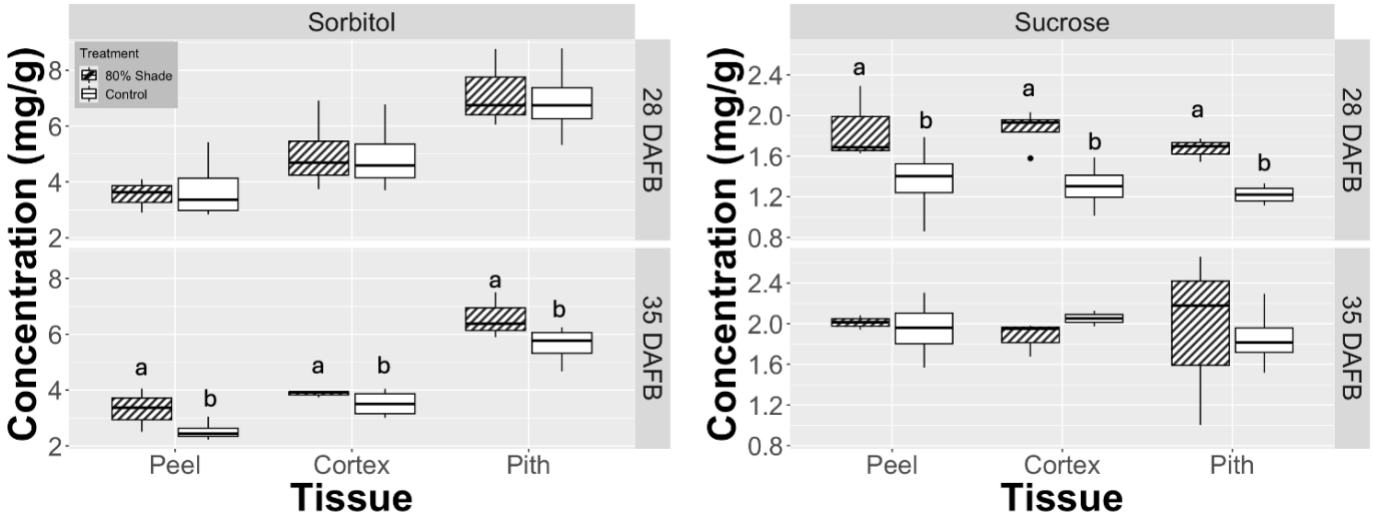

Figure 3. Sorbitol and Sucrose concentrations for early and mid-fruit development in peel, cortex, and pith tissues of 'Red Delicious' fruit. Similar letters above the bars indicate lack of significant differences between treatments, within a given date. Metabolites were measured using gas chromatography, and concentrations were calculated using Phenyl ß-D-glucoside as a standard.

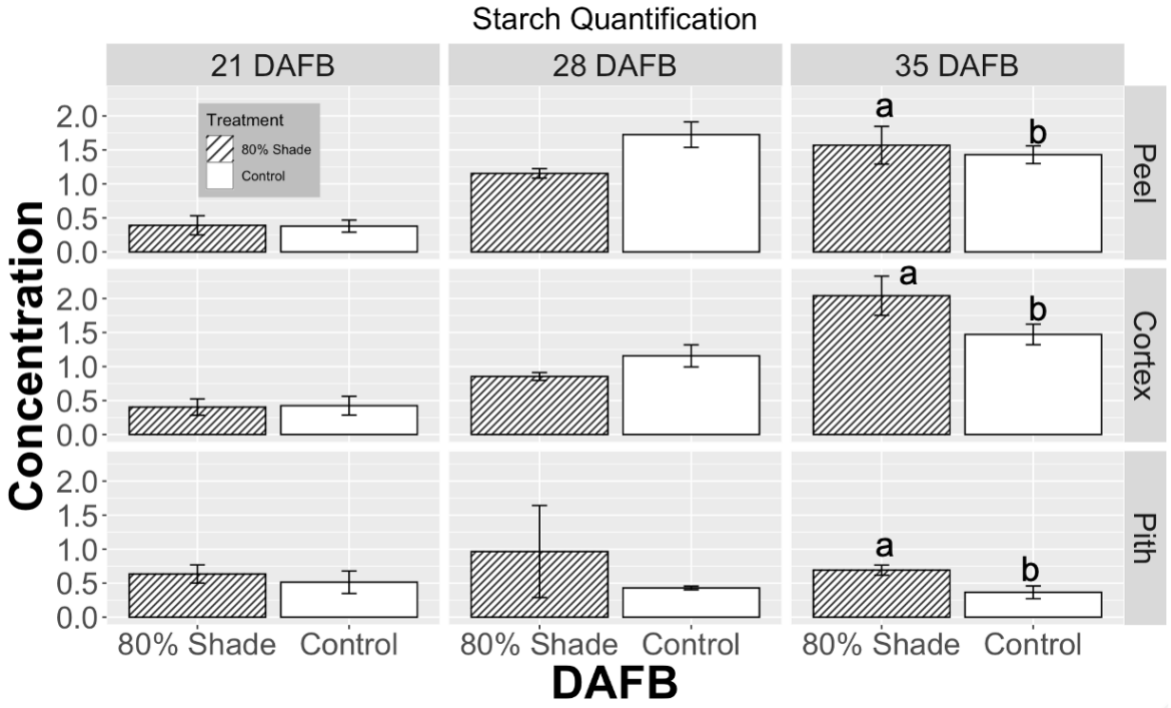

Figure 4. Starch quantification of peel, cortex, and pith 'Red Delicious' tissues from early to mid-fruit development (21, 28, and 35 DAFB). Similar letters above the bars indicate lack of significant differences between treatments by ANOVA tests.

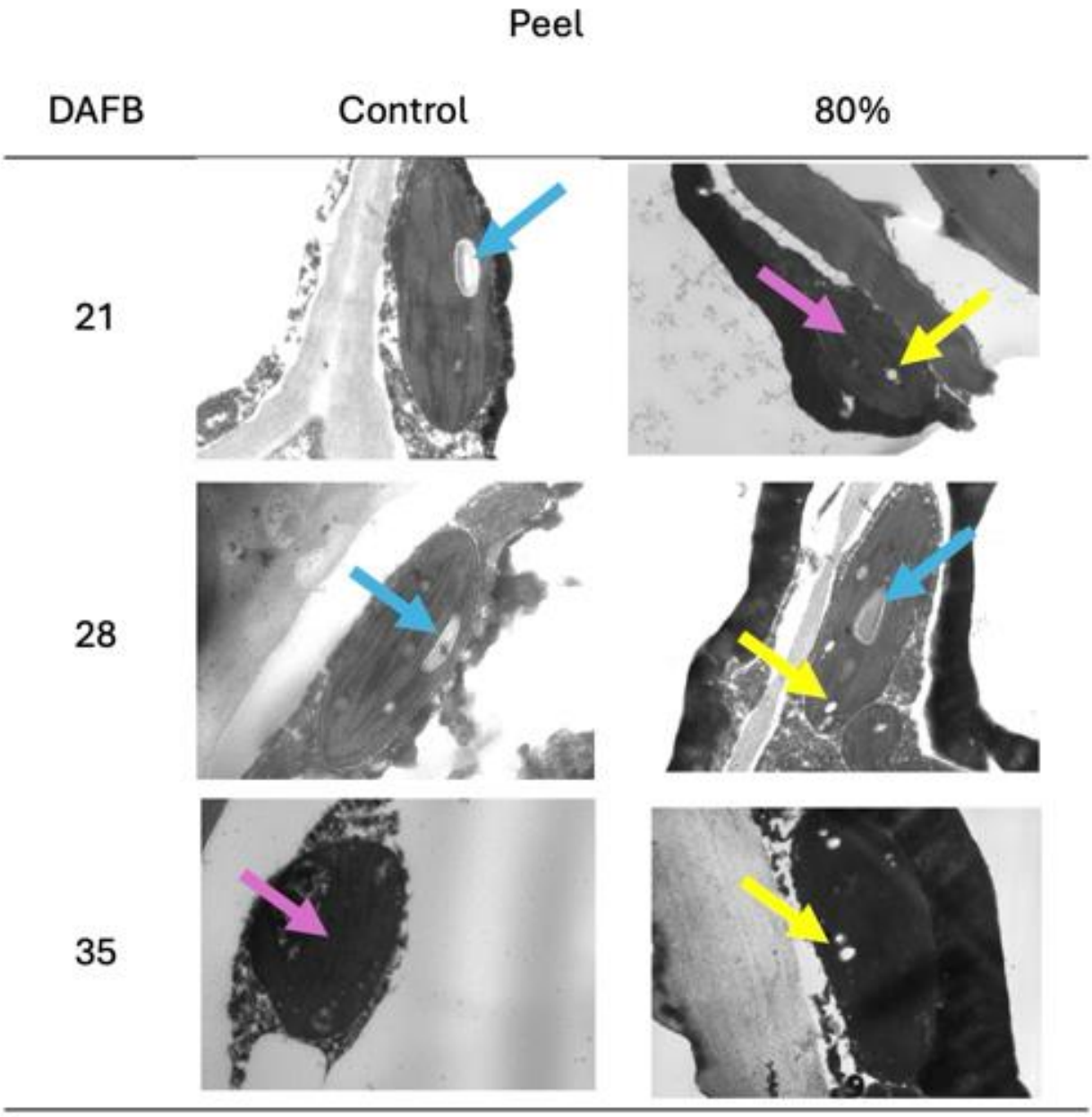

Figure 5. Time series of ultrastructural plastid features in peel tissue in control and 80% shaded fruit 21, 28, and 35 DAFB. Starch granules (blue arrows), grana (purple arrows), and plastoglobules (yellow arrows) were noted across all stages. Images were taken at high magnification on a JEOL JEM 1011 TEM at 100 kV.

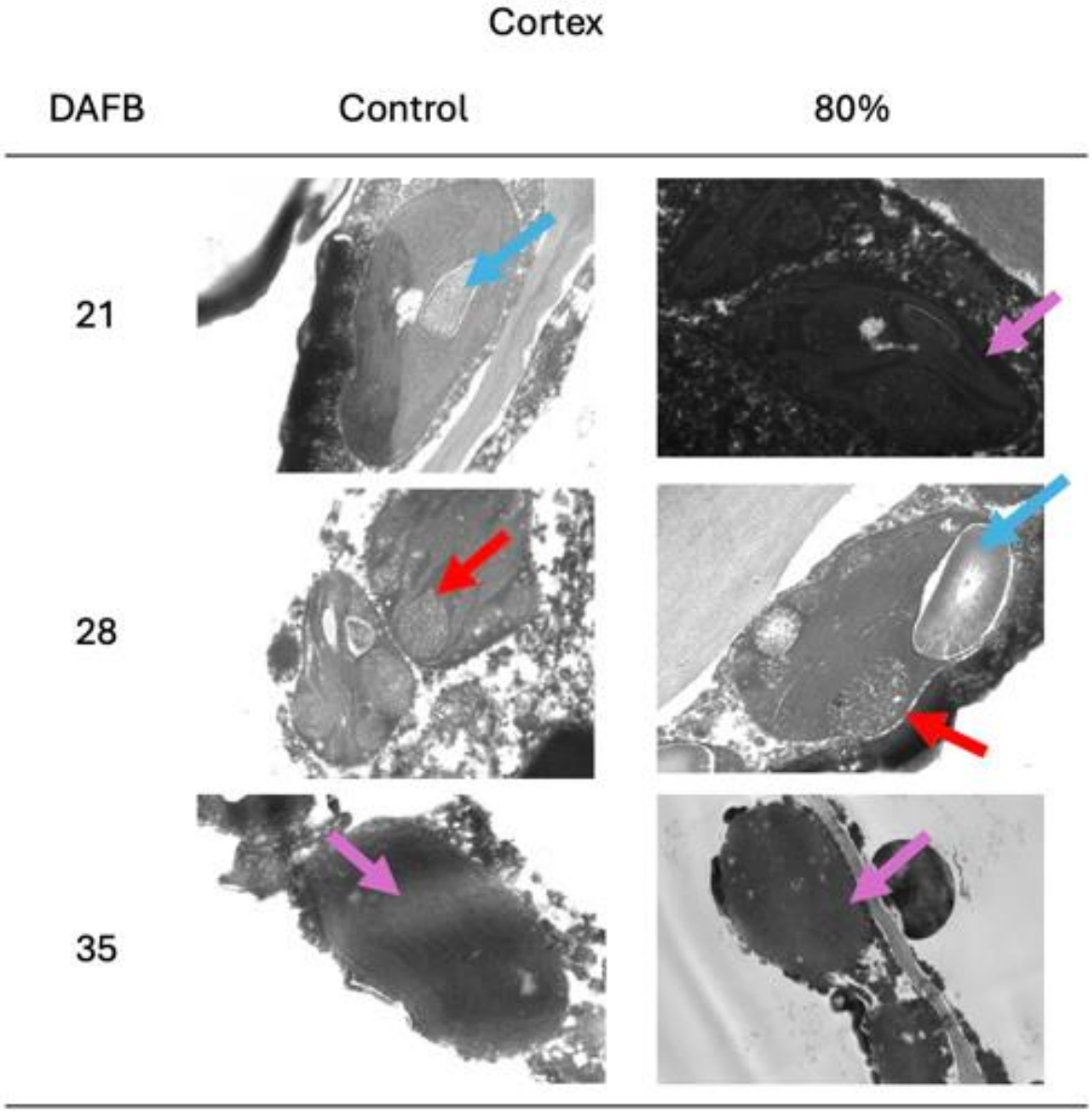

Figure 6. Time series of ultrastructural plastid features in cortex tissue in control and 80% shaded fruit 21, 28, and 35 DAFB. Starch granules (blue arrows), grana (purple arrows), and inclusions (red arrows) can be observed in both treatments. Images were taken at high magnification on a JEOL JEM 1011 TEM at 100 kV.

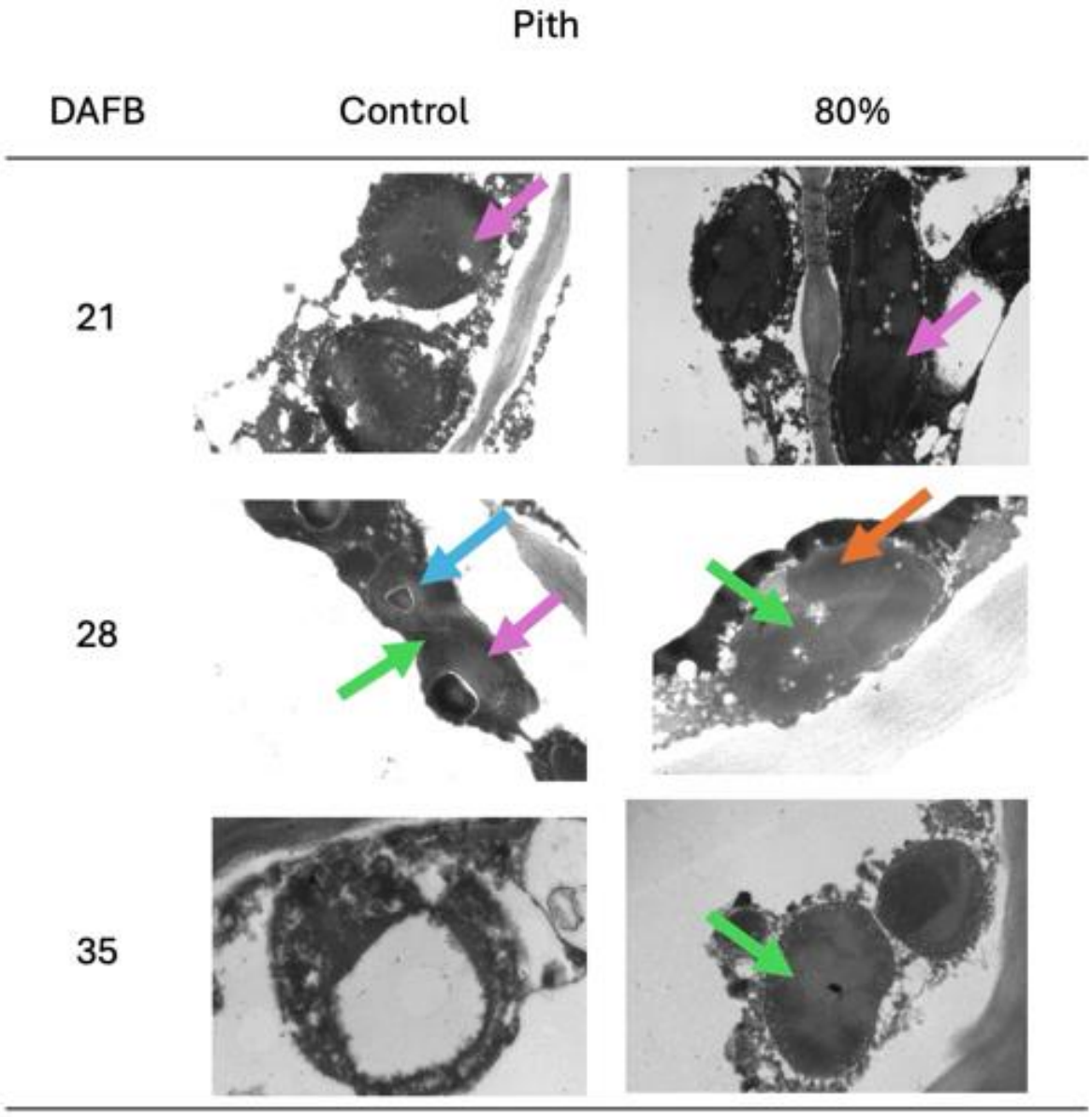

Figure 7. Time series of ultrastructural plastid features in pith tissue in control and 80% shaded fruit 21, 28, and 35 DAFB. Starch granules (blue arrows), grana (purple arrows), osmiophilic bodies (orange arrows), and prolamellar bodies (green arrows) can be observed in both treatments. Images were taken at high magnification on a JEOL JEM 1011 TEM at 100 kV.

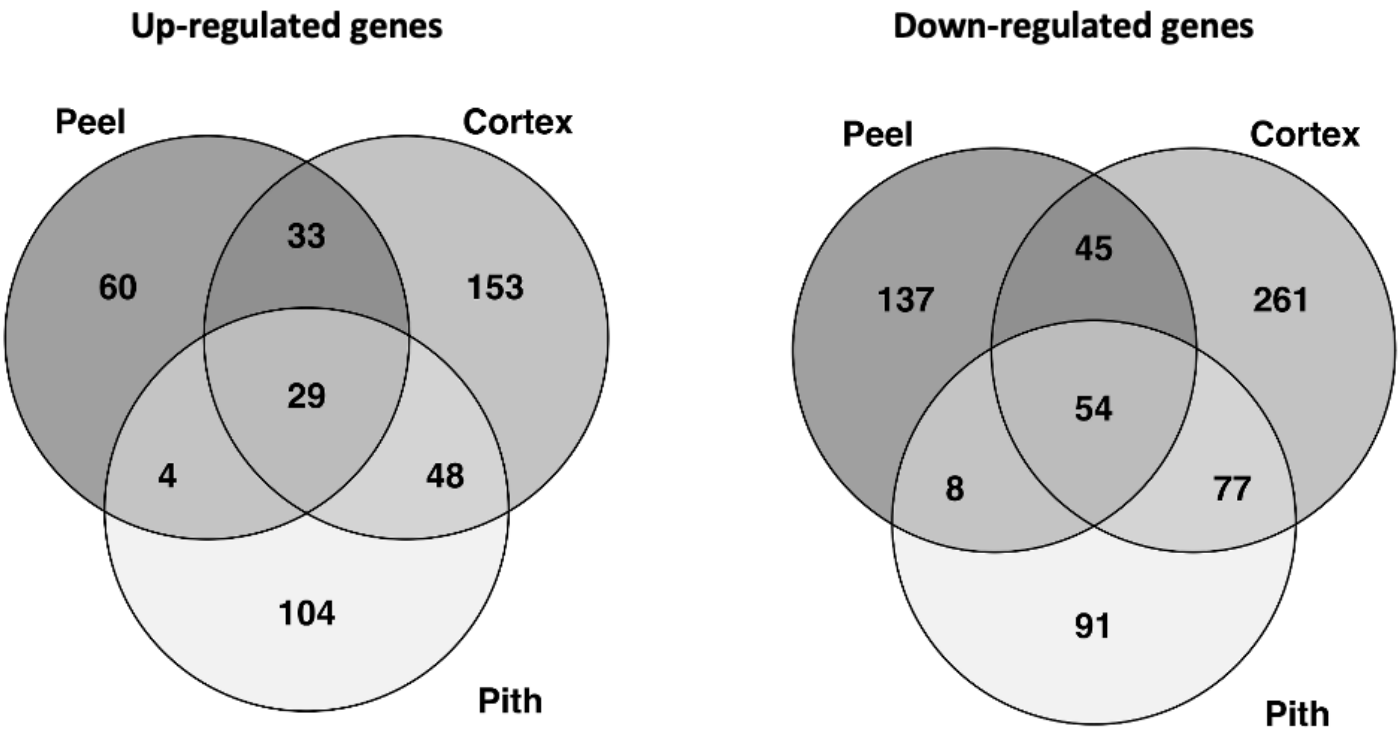

Figure 8. Venn diagrams displaying the total number of differentially expressed genes in the peel, cortex, and pith tissues of shaded fruit regardless of Log_2fold change. The control fruit transcriptome was used as a reference in the calculation of up/down regulated shaded fruit transcriptome. The DESeq2 program was used to calculate differentially expressed genes with adjusted p-value of less than 0.05

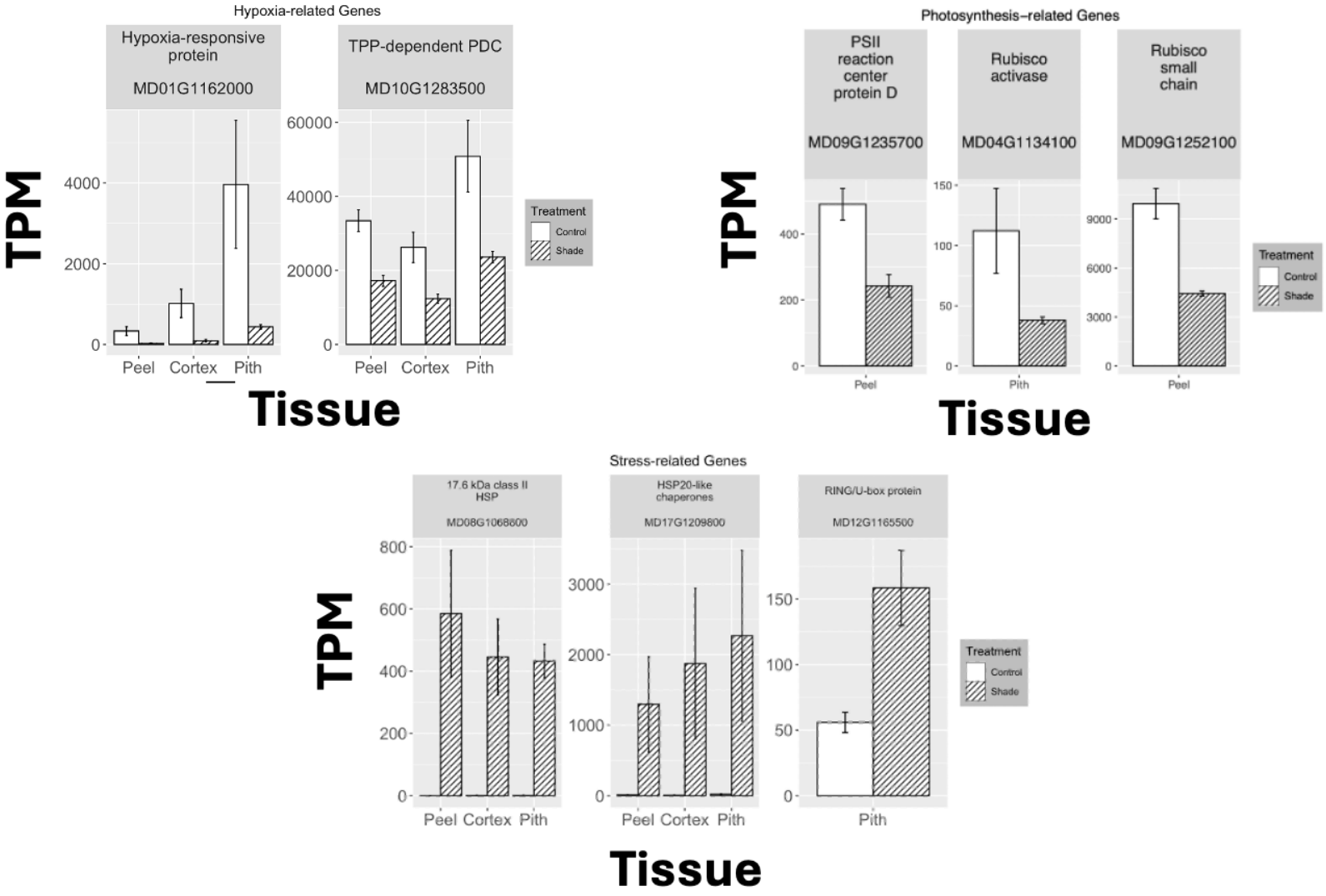

Figure 9. Examples of differentially-expressed genes in the peel, cortex, and pith tissues between control and 80% shaded fruit. Processes range from photosynthesis, hypoxia, and stress in early-fruit development. Data are presented in transcripts per million (TPM) as determined by DESeq2.

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CHAPTER 3

FRUIT GROWTH RATE

INTRODUCTION

Overall fruit growth rate is determined by a combination of several elements including genetic, environmental, and orchard management factors. During early fruit growth, the ability of a fruit to acquire resources as a sink defines its ability to continue growth and maintenance respiration (Baïram et al., 2019). The allocation of nutrients to different plant organs relies on physiological factors to adjust and compete for limited resources in the beginning of fruit, shoot, and root development (Falchi et al., 2020). Sugars produced by the leaves are loaded into the phloem *via* an apoplastic or symplastic route that may involve transporter proteins like SWEETs and are then transported along a high to low carbohydrate gradient in the phloem that is maintained by rapid metabolism of carbohydrates into vacuoles (Braun, 2022; Milne et al., 2018). Unloading of sugars into sinks such as apple fruit is thought to be achieved via the apoplastic pathway, with active transport of sugars across membranes using sugar alcohol, hexose, and sucrose transporters (L.-Y. Zhang et al., 2004). Some fruits such as tomato, grape and jujube have been documented to phloem unloading pathways along different phases of development from symplastic to apoplastic or *vice versa* (Ma et al., 2019). Because the remobilization of stored reserves of non-structural carbohydrates is controlled by a plant organ's sink strength, apples are partially sink-limited during the beginning stages of growth when most of the carbohydrates available are being allocated to new shoots (Cai et al., 2021).

However, apple fruits are also partially source-limited, with thinning increasing fruit growth as well. Without enough resources to fully support each fruit, many will drop from the tree. These source-sink relationships of fruit are also heavily influenced by the position of fruit in the cluster of flowers such that generally, the king fruit preferentially receives more resources as it blooms slightly earlier and has an advantage over lateral fruits (Ferree et al., 2001). Further, the laterals that bloom first will have an advantage over lower laterals that bloom later. Thus, the physiological aspects in relation to source-sink interactions influence much of the growth rate of the fruit. Environmental factors and management practices such as pruning, watering, and light availability affect the growth of the fruit, but it is the source-sink relationships that act as a dial to distribute the carbohydrate production under these conditions. In this way, perennials like apple trees have different carbohydrate usage strategies that focus on conservative use of resources in comparison to annuals which may be forced to grow faster and not reserve carbohydrates for the next growing season (Burnett et al., 2016).

During early fruit growth, the first 30 days of growth is facilitated by cell division. The transcription of cell-cycle related genes such as *CYCLIN DEPENDENT KINASES*, *CYCLINS*, and various other cell-division related genes like WEE kinases encourages cell division (Pettkó-Szandtner et al., 2015). CYCLIN DEPENDENT KINASES phosphorylate important target proteins to act as checkpoints and allow the cell cycle to move on to the next phase (Tank & Thaker, 2011). The cell cycle consists of G1, S, G2, and M phases, where the concerted action of CDKs and cyclins are essential for successful completion of the cell cycle (Inzé & De Veylder, 2006). This extremely high rate of cell division requires a large input of carbohydrates to support the early stages of growth. After the cell division stage of fruit development is complete, the fruit

volume can grow to 25-30x its original size in the case of ‘Royal Gala’ and ‘Scifresh’ apples during cell expansion when cells are amorphous and easily expanded (Ng et al., 2013). To best evaluate fruit growth potential, it is often necessary to remove restraints imposed by positioning on the fruiting cluster as well as competition among fruit, thereby allowing for minimizing source limitations on growth.

This source-sink relationship in conjunction with its ability to provide sugars to support cell division and subsequent cell expansion will impact the relative growth rate of fruits. In one study, thinned apple fruit were found to have a 39% higher RGR than non-thinned fruits up until June, and fruits maintained this significantly higher rate for almost the entire duration of the fruit season. After a slight reduction in differences in June, RGR values were almost never less than 20%, but thinned fruit still maintained a higher RGR (Reyes et al., 2016). Researchers ascribed this to the increased photosynthates available after shoots are finished growing in the summer. The ability of a single fruit to quickly gain strength as a sink while being uninhibited by other fruitlets will allow them to effectively reach their growing potential after the process of cell division has impacted the potential of fruit size the most (Archbold, 1992). Indeed, fruits appearing on the slow-growing end of a bi-modal distribution representing growth rate were consistently the majority of fruits to abscise (Zibordi et al., 2009). Thus, the growth rate of fruitlets can also be significant indicators of its potential to stay on the tree.

Measuring growth of fruits and plants overall has usually been done using the relative growth rate (RGR) instead of the absolute growth rate (AGR) when desiring to look at a standardized unit. In some cases, ontogenic drift can misrepresent growth rate if size-dependent characteristics are not taken into consideration, as certain traits change over the

course of a plant's life cycle (Wright and Mcconnaughay, 2002). By and large, using RGR allows scientists to compare measurements of different fruits or tissues and draw conclusions as it is a measurement of a growth trait per unit of plant material per unit time, and it based off of the following formula: $\frac{(lnD_2 - lnD_1)}{(T_2 - T_1)}$ where D2 and D1 represent a fruit growth metric (E.g.: diameter) at times T1 and T2 respectively (Radford, 1967). High correlation between the fruit RGR and the way that the biomass is accumulated, such as through lignocerate, stearate, or palmitate fatty acids present in membranes, has been noted. It is suggested that the composition of cell walls can give an indication of the growth rate of fruits through the amount of arabinose, glucose, or fucose in apples (Roch et al., 2020). It has even been shown that the RGR of a fruit varies within the different fruit tissues itself, with the endocarp having a higher RGR than other parts of the mesocarp and epicarp in peaches during the first phase of growth (Baldicchi et al., 2015). Also, in apple, RGR of the cortex is greater than that of the pith during early fruit development (Jing and Malladi, 2020). Measuring and sampling the cortex of the apple fruit to determine its RGR would be an ideal way to track the transcriptional changes associated with differential fruit growth under source non-limiting conditions. Thus, analyzing the transcriptome of fruits with high growth rates could reveal key genes that facilitate growth.

MATERIALS AND METHODS

Relative growth rate analysis

The study was conducted in 2022 at the University of Georgia Mountain Research and Education Center in Blairsville, Georgia at two timepoints that spanned four days each. The first timepoint was 17-21 DAFB and the second timepoint was from 49-53 DAFB. For both

experiments, 12 'Empire' trees were divided into four blocks that functioned as replications with three trees comprising one experimental unit. Ten individual fruit on each tree were randomly selected ensuring only one fruit per cluster, tagged by numbering each fruit. Each fruit had two diameter measurements taken along perpendicular axes using digital calipers. Four days after initial tagging, the diameter measurements were taken again, and all the fruit were collected.

Fruits were harvested, wrapped in a wet paper towel, and brought inside a laboratory space. The fruits were then sliced on the transverse plane to obtain a radial section from which cores of the cortex were sampled using biopsy punches. The cortex cores were stored individually and snap-frozen in liquid N. An extra thirty fruit were taken from Blairsville at the second collection date and wrapped in moist paper towels to bring back to the University of Georgia, Athens, GA. These fruits were sliced in half from the pedicel end to the calyx end and scanned onto a computer using an Epson Perfection V600 Photo (Los Alamitos, CA, USA). ImageJ was then used to measure the diameter and length of each fruit in pixels at a resolution of 600 dots per inch. The measurements in pixels were then converted into mm using the equation:

$$mm = \ number\ of\ pixels * 0.0423333$$

The resulting diameter and length measurements in mm of the thirty fruit were plotted in Excel to determine the relationship between fruit diameter and length. The average diameter measurements (of two readings) for each fruit were used to estimate fruit length. Subsequently, the diameter and the estimated length measurements of individual fruit were used to calculate the total volume of each fruit using: $\frac{(3.1416*(D^2)*L)}{6}$ where D and L represent either the initial (D_1 or L_1) or final (D_2 or L_2) measurements of the fruit. The volume data of each

individual fruit was then used to determine the RGR as: $RGR_{vol} = \frac{(lnV_2 - lnV_1)}{(T_2 - T_1)}$ where V_2 and V_1 represent the final and initial volumes, respectively, and T1 and T2, represent initial and final times, respectively (4 d). Fruit rates that exhibited negative growth rates due to errors in measurement were removed from the data analyses. Any outliers as determined by boxplots were also removed from the dataset before proceeding with further analyses.

An inverse relationship between the initial volume of the fruit and the relative growth rate of the fruit was noted only for the 17-21 DAFB samples, suggesting that the RGR was, in part, influenced by the initial volumes (R^2=0.244; P-value < 0.001). To overcome the effects of this relationship and to standardize initial fruit size, quartile analyses were used to reduce the spread of initial fruit size data. The QUARTILE.EXC function in Excel was used to find the 25%, 50%, and 75% quartiles of each of the four replicates in 17-21 DAFB. Data within the 25%-75% quartile range was used for further analysis. These fruits were sorted based on their RGR values. From this sorted list, three fruit with the highest RGR and three fruit with the lowest RGR were grouped into high RGR and low RGR groups, respectively. The differences within and among replicates were examined using T-tests.

The data for the 49-53 DAFB samples did not exhibit the same ontogenic drift relationship between initial volume and RGR. The fruit volume data was processed by removal of statistically determined outliers. Similarly to the 17-21 DAFB samples, the fruit exhibiting the highest and lowest RGR values were chosen for further analyses.

RNA Extraction

Total RNA extractions were performed slightly differently for 17-21 DAFB and 49-53 DAFB samples. There was not enough tissue for the 17-21 DAFB samples to perform RNA extractions for individual fruit. Instead, any available ground tissue (approximately 30 mg each) from the 3 fruits with the highest and lowest RGRs were pooled for each replicate. Total RNA was extracted from these pooled tissues. For the 49-53 DAFB samples, there was enough tissue to complete the six individual RNA extractions (three for high RGR and three for low RGR) per replicate. The pooled 17-21 DAFB RNA that was prepared for sequencing was comprised of around 100 ng of RNA from each of the three highest or lowest samples. Regardless of the pooling methods, the RNA extraction was completed using the same protocol as per Jing and Malladi (2020).

RNA-Sequencing, differential gene expression, and GO analysis

Illumina paired-end strand-specific eukaryotic mRNA (polyA enrichment) total RNA sequencing was performed by Novogene (Novogene Corporation Inc., Sacramento, CA, USA). Raw reads were downloaded from the Novogene server and uploaded into the UGA Sapelo2 cluster. FastQC reports were obtained using FastQC/0.11.9-Java-11 to run initial quality control checks on raw reads, and adapters were trimmed using the paired-end option of Trimmomatic version 0.39 (Bolger, A. M., Lohse, M., & Usadel, B. 2014). After adapter trimming, another QC report was run before STAR alignment. Version 2.7.10b of the STAR alignment program was used to map reads against the GDDH13 genome and gene models downloaded for the same genome reference from the Rosaceae Genome Database (Jung et al. 2015). After all reads were

mapped, each sample file generated a “readspergene.out.tab” file that was exported to Excel and used to perform a differential gene expression analysis comparing the low RGR and high RGR samples. The reference was set to the high RGR samples, so that the differentially expressed genes are in comparison to the fruits with the highest growth rates.

After obtaining a list of differentially expressed genes from the STAR alignment program, we used AgriGOv2 (https://systemsbiology.cau.edu.cn/agriGOv2/index.php) to perform a GO analysis on the upregulated and downregulated genes. A single enrichment analysis (SEA) was done using the *Malus x domestica* Rosaceae species option. The GDDH13 V 1.1 by Blast2GO was selected as the background reference, and our list of differentially expressed genes that were upregulated or downregulated for 17-21 DAFB and 49-53 DAFB were used as queries. The direct acyclic graph (DAG) trees were used to consider biological processes that displayed significant GO enrichment.

RESULTS

Relative growth rate analysis

The RGR analysis for the 17-21 DAFB data yielded only three usable replicates. One replicate was non-normal and deviated substantially from the fruit size data of the other three replicates and was therefore not used in the analysis. However, all four replicates for the 49-53 DAFB sampling was used. Within both collection dates, there was no statistical difference in the initial volumes between the high and low RGR fruit that were used in the proceeding analyses. However, there was still a statistical difference between the RGR_{vol} measurements. Thus,

confirming that the fruit started off as statistically the same volume yet exhibited different growth rates between the initial and final measuring dates.

The fruits collected for the 17-21 DAFB sampling date represent the cell division phase of development, and the fruits collected for the 49-53DAFB sampling date represent the cell expansion phase. The initial volume for 17-21 DAFB fruits used in the RNA sequencing analysis was 6.872 ± 0.158 for the low RGR category and 6.67 ± 0.21 for the high RGR category. These initial volumes were not statistically significant, but the growth rate values for the low and high RGR groups were significant ($p < 0.0061$). The low RGR value was 0.138 ± 0.011 and the high RGR value was 0.202 ± 0.018. Thus, we ensured that our downstream RNA sequencing analysis would be performed to accurately observe differences in fruits with genetic patterns that contributed to the increased growth even when the fruits all began at the same size. The samples used for the 49-53 DAFB RNA sequencing also followed this pattern. Initial volumes for the low and high fruits were 9.794 ± 0.311 and 9.786 ± 0.433, respectively. RGR values for the low and high fruits were 0.034 ± 0.433 and 0.06 ± 0.006. The RGR values were significantly different between groups ($p < 0.00038$) while the initial volumes were not significant.

The RGR_{vol} clearly decreased over time when comparing the 17-21 DAFB RGR_{vol} values to the 49-53 DAFB RGR_{vol} values. The 49-53 DAFB samples were collected after the period of early fruit development where cell division is highest, so the growth rate demonstrated that through a sharp decrease in RGR values across samples. The initial volumes of 49-53 DAFB samples were much higher than the 17-21 DAFB samples, since the period of cell expansion would coincide with the 49-53 DAFB collection time, these fruits were much larger later in the growing season.

Altogether, the data accurately reflected the growth stages that the fruit were in, and the fruits with the highest and lowest RGR values were used in the next phase of RNA sequencing.

RNA Sequencing Bioinformatics

Using the bioinformatics pipeline, we identified a total of 204 differentially expressed genes in the 17-21 DAFB and 49-53 DAFB sequenced samples. All samples were analyzed using the high RGR samples as the reference. Therefore, all findings are with respect to the samples with the high growth rate.

In the 17-21 DAFB samples, a total of 130 genes were differentially expressed with an adjusted p-value of <0.05, with 27 being upregulated and 103 being down regulated. After being run through AgriGO, out of the upregulated 17-21 DAFB genes 20 were annotated in the query list and there were no significant GO terms. In comparison, the downregulated 17-21 DAFB samples had 67 annotated in the chosen background and there was a total of 50 significant GO terms. These GO terms were associated with several downregulated processes with some of the categories including DNA conformation change, chromatin assembly, protein-DNA complex assembly, and nucleosome organization and assembly. Genes that related to some of these categories consisted of histone family proteins MD03G1023100, MD01G1124600, and MD11G1027400, with $\log_2$fold changes of -1.456, -1.207, and -1.185. Cyclin and cyclin-dependent kinases MD15G1344200 and MD04G1180000 were also downregulated with $\log_2$fold changes of -1.025 and -1.064. *DNA-BINDING HORMA FAMILY PROTEIN (*MD16G1109600*)* also had a $\log_2$fold change of -1.021, and others that function within

cell division processes like the PLATZ TRANSCRIPTION FACTOR protein (MD16G1015800) had a change of -1.422.

The 49-53 DAFB samples had far fewer significant GO terms overall. In the 49-53 DAFB samples, a total of 74 genes were differentially expressed, with 33 being upregulated and 41 being downregulated. The upregulated 49-53 DAFB samples had only 26 genes annotated compared to the background we chose, and of those 26 there were no significant GO terms associated with the list. The downregulated genes had 24 annotated genes out of the query list, and 3 of those were significant GO terms. The graphical tree displayed groupings of differentially expressed genes associated with processes like single organism metabolic process and oxidation-reduction process. Downregulated genes that were sorted into these groupings according to AgriGO have descriptions such as being associated with *CYTOCHROME P450* (MD01G1172500), *FATTY ACID DESATURASE 2* (MD14G1058300), *PEROXIDASE SUPERFAMILY* PROTEIN (MD12G1184600), *HYDROXY METHYGLUTARYL COA REDUCTASE 1* (MD15G1227900), *and CLASS II DAHP SYNTHETASE FAMILY PROTEIN* (MD15G1372600). The $\log_2$fold change of these downregulated proteins was much more variable, with values of -0.437, -1.545, -0.658, -0.328, and -0.234, respectively.

DISCUSSION

The differential gene expression in the 17-21 DAFB samples was predominantly associated with cell division pathways and DNA organization. Because the 17-21 DAFB sampling

occurred during early fruit development, the results of the RNA sequencing likely corresponded to the expected expression of cell division-related genes.

The fruits with a low RGR had decreased gene expression related to primarily to histones, cyclins, and cyclin dependent kinases, which implies that the fruits with a higher RGR display higher or extended cell production activity. Being in a state of cell proliferation is dependent upon many genes that operate within chromatin dynamics and cell cycle function (Desvoyes et al., 2010). This is appropriate considering the increase in cell division would require first a relaxation or adjustment of the tight coiling that histones manage so that the DNA may be accessed. Chromatin dynamics have tight control over the developmental program of an organism through the acetylation and deacetylation of its histones that regulate its gene expression (Rosa et al., 2015). After ensuring access to the DNA, the process for cell division would be allowed to begin with the recruitment of transcriptional complexes like polymerases and the cell cycle would continue with the proper functioning of checkpoints like CDKs. Core histone proteins are associated with packaging of newly synthesized DNA into nucleosomes. Hence, existing histones and newly synthesized histones are critical in ensuring proper DNA packaging during cell division.

In samples that had a low RGR in comparison to the samples with a high RGR, the *DNA-BINDING HORMA FAMILY PROTEIN* was noted to have been downregulated during the 17-21 DAFB samples with a $\log_2$fold change of -1.021. The HORMA (Hop1, Rev7, and Mad2) domain is responsible for many molecular roles that maintain and monitor the state of the cell cycle. It manages DNA repair pathways and monitors spindle assembly by acting as a checkpoint to ensure the proper function of the cell cycle (Rosenberg & Corbett, 2015). Similarly, *GAMMA*

HISTONE VARIANT H2AX was downregulated (log_2fold change of -1.319) in low RGR samples, which suggests that slow-growing fruitlets do not require as much DNA repair activity, as GAMMA H2AX functions in damage signaling and the repair of double-stranded breaks (Fan et al., 2022). The *DP-E2F-LIKE 1* (MD06G1181700) was also downregulated (log_2fold change of -1.009) in low RGR fruits, and this protein is expressed in actively dividing cells as a transitional checkpoint before duplicating genetic material in the G1/S transition (Vlieghe et al., 2005; W.-F. Lam & La Thangue, 1994). It appears that fruits with higher RGR can facilitate growth responses better than low RGR fruit owing to higher or extended cell production activity.

As fruits continued to develop, there were fewer genes that were significantly different between the high and low RGR samples as indicated in the 49-53 DAFB collection date. This would correspond to the time when cell division is finishing up and the next phase of cell expansion is beginning to take over. The significant DEGs that had log fold changes of >1 were not specifically attributed to any one process as there were only 7 downregulated genes and 9 upregulated genes that had a large log fold change. Two of the downregulated genes had descriptions of transport of efflux, while another two had descriptions of terpene synthase or fatty acid desaturase. The genes that are upregulated in the low RGR 49-53 DAFB samples were related to *EXPANSIN A4* and *B-GALACTOSIDASE 12*, indicating the tissue's shift in metabolic program into one of expansion. One possibility is that earlier exit from cell production and progression through the cell expansion phase may result in higher expression of the cell expansion-related genes in the low RGR fruit. Additionally, the genes that were differentially expressed in May seemed to have primarily reduction-oxidation functions and were more focused on the metabolic side of fruit growth. The *FATTY ACID DESATURASE (FAD) 2*

(MD14G1058300) with a log_2fold change of -1.545 could be impacting growth through cell membrane composition (Berestovoy et al., 2020). In olives (*Olea europaea*), FADs regulate many functions that are tissue-dependent and influence fruit development, thylakoid formation, wound responses, and environmental stressors like cold, salt, and drought conditions (Niu et al., 2022; Poghosyan et al. 1999). Reducing expression of critical redox-related proteins like FADs could be impacting the structural integrity of membranes.

These differences between the transcriptomic profile of fruits with higher and lower rates of growth highlight the genetic factors that affect fruit growth without the competition between other fruits in the cluster. It suggests that having control over gene expression through the transcription of histones to monitor chromatin access is more influential to the growth patterns of fruit than other physiological processes in early fruit development. Having ample production of proteins that regulate critical checkpoints in the cell are also important. These differences in expression of cell cycle genes diminish over time as cell division slows and cell expansion becomes more apparent. Potentially, the main factors influencing growth rate during the middle phase of fruit development shift to a metabolic perspective with control over redox reactions becoming increasingly important to the growth of the fruit. Being able to efficiently utilize carbohydrates and synthesize appropriate fatty acids is more critical to the growth of fruit as it supports the beginning phases of ripening.

CONCLUSION

The differential gene expression and GO analyses reinforced concepts relating to cell growth and fruit development patterns that have been documented previously. The most

genetic variation in terms of genes that contribute to a difference in growth rates can be observed in the earlier time point, which was 17-21 DAFB in 17-21 DAFB. There were markedly fewer DEGs in the 49-53 DAFB samples that would represent the middle phase of fruit development. Genes that were found 17-21 DAFB were mainly related to cell division, with histones and cyclins being a predominant category of genes that we saw throughout the analysis as having the biggest log fold changes. Interestingly, there were no significant GO terms that were upregulated in any of the 17-21 DAFB or 49-53 DAFB samples. The differences in gene expression came solely from the down regulation of genes in both groups of samples, suggesting that fruits with lower growth rates do not express critical genes in high volume. Altogether, this reinforces the notion that cell division is the most critical factor in final fruit size, as it was the predominant category in the DAG tree for the 17-21 DAFB samples. It does shed light on the importance of histones within this process of cell division and may be the focus of genetic research in the future to produce larger fruits.

FIGURES

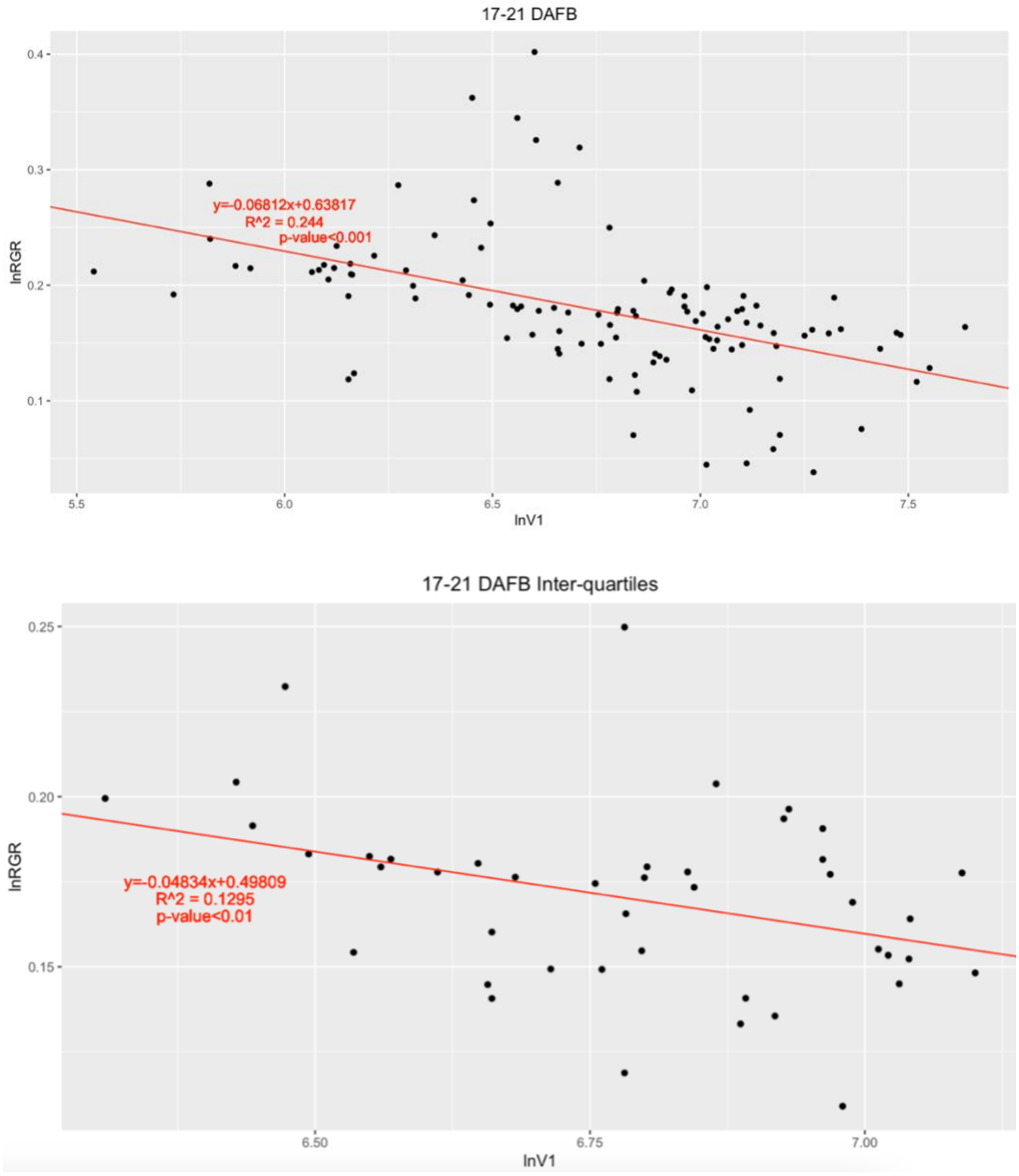

Figure 10. Fruit measurements from 17-21 DAFB early fruit development data showing the relationship of initial volume to RGR without (top) and with (bottom) inter-quartile range filtering to reduce the extreme spread of data.

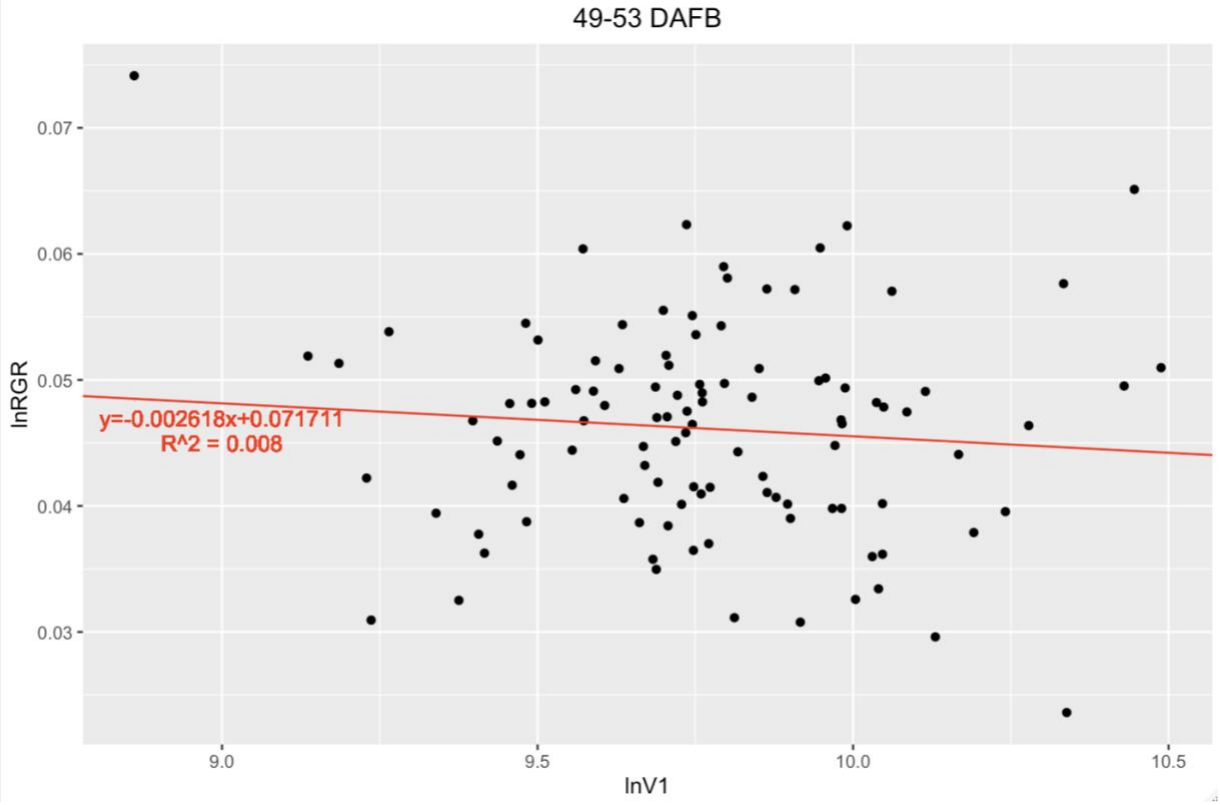

Figure 11. Fruit measurements from 49-53 DAFB mid fruit development showing the relationship of initial volume to RGR. Filtering was not needed as the relationship between initial volume and growth rate were not significant.

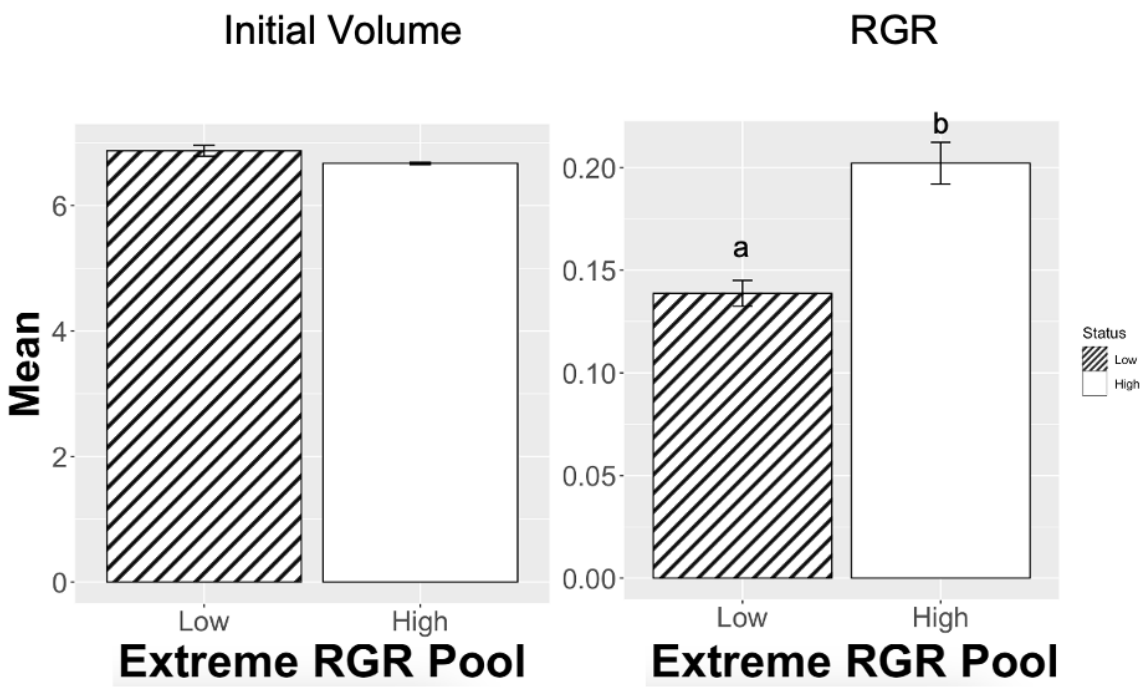

Figure 12. Data for fruit at 17-21 DAFB in early fruit development showing that the RGR populations are NS for initial volume but statistically differ for RGR measurements. Letters above the bars indicate significant differences between the high and low RGR populations.

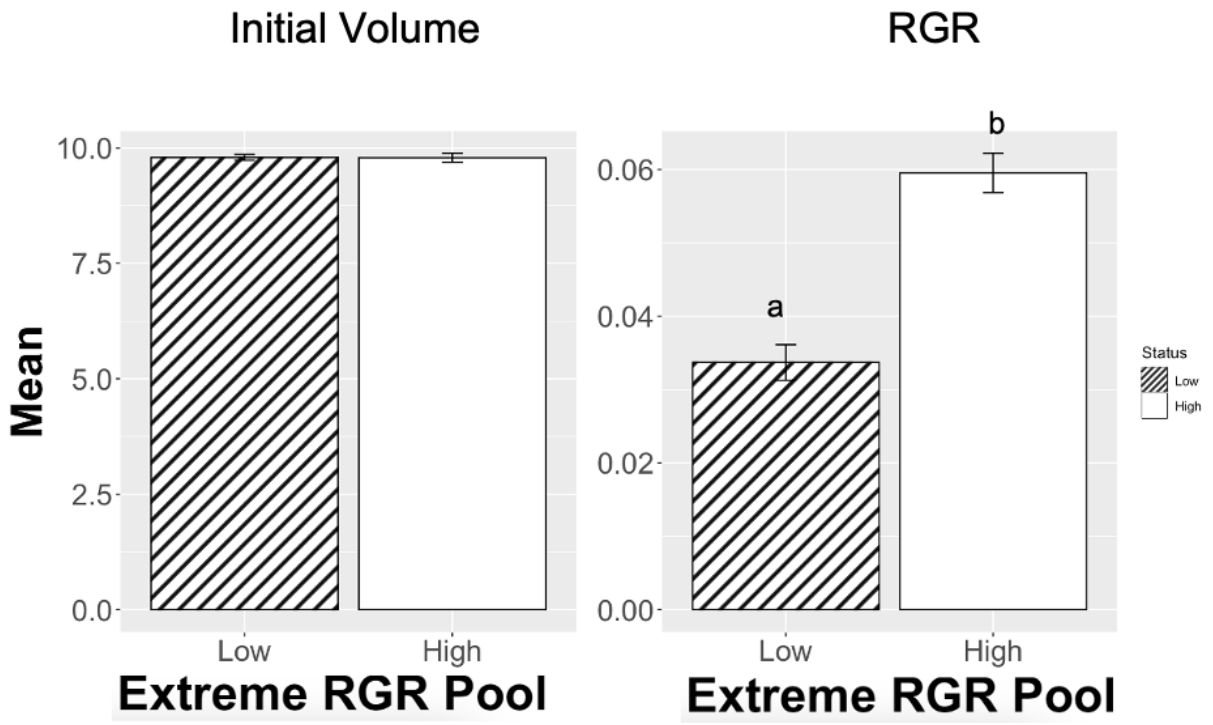

Figure 13. Data for fruit 49-53 DAFB in early fruit development showing that the RGR populations are NS for initial volume but statistically differ for RGR measurements. Letters above the bars indicate significant differences between the high and low RGR populations.

Figure 14. DAG tree for fruit 17-21 DAFB in early fruit development between high and low RGR fruits. The most significant pathways were associated nucleosome assembly, chromatin assembly, and protein-DNA assembly. Solid black arrows indicate one process is a part of another, green arrows indicate negative regulation, and dotted arrows indicate two significant nodes. Red color indicates highly significant processes with orange being less significant.

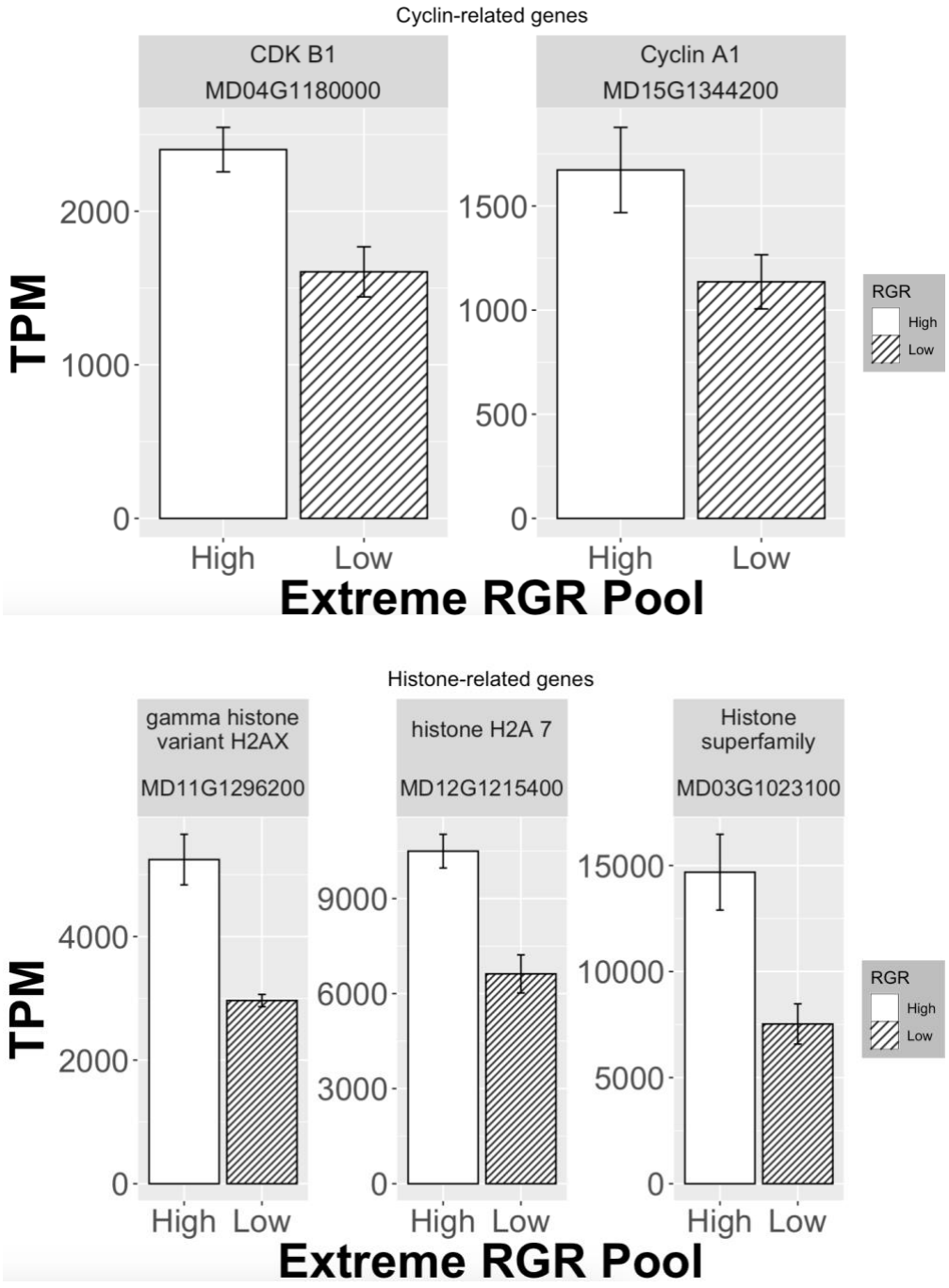

Figure 15. Examples of cyclin and histone-related genes that are differentially expressed between high and low RGR fruit 17-21 DAFB in early fruit development. Data are presented in transcripts per million (TPM) and are derived from the DESeq2 transcriptome analyses.

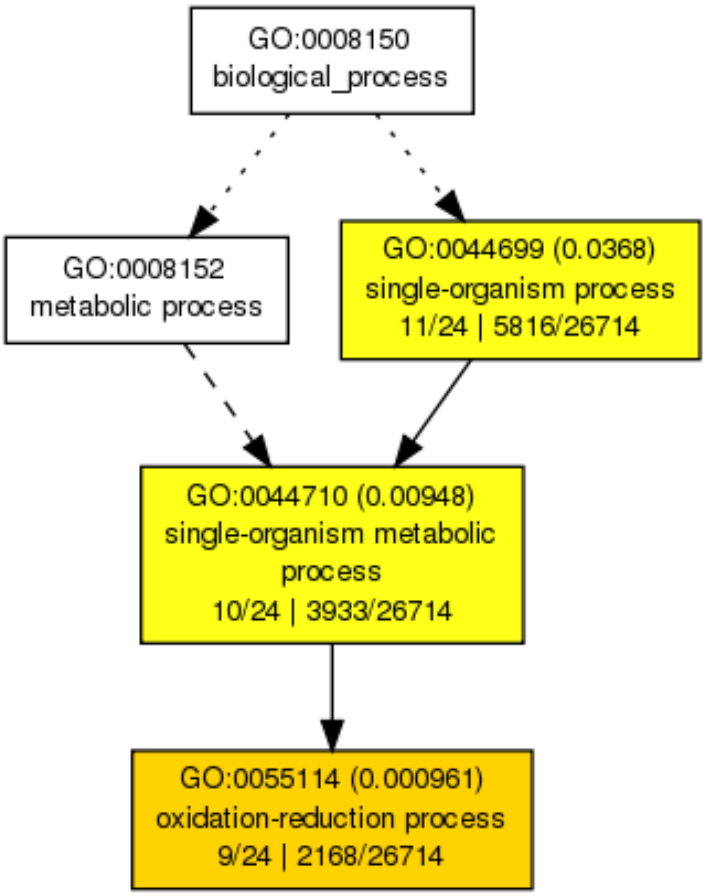

Figure 16. DAG tree for pathways that are significantly different between high and low RGR fruits 49-53 DAFB in mid fruit development. The most significant pathway was associated with oxidation-reduction processes. Solid black arrows indicate one process is a part of another and dotted arrows indicate two significant nodes. Color gradient indicates varying levels of significance with less significant processes being white and more significant processes turning orange.

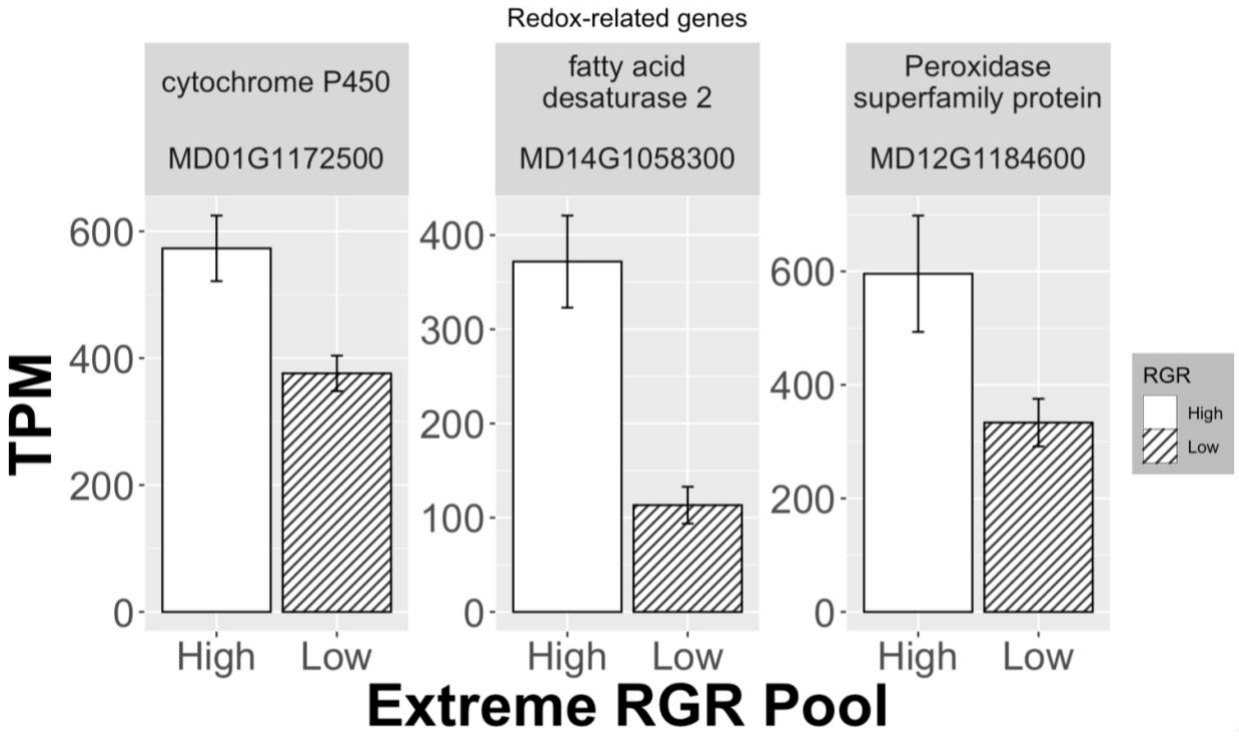

Figure 17. Examples of redox-related genes that are differentially expressed between high and low RGR fruit at 49-53 DAFB in mid fruit development. Data are presented in transcripts per million (TPM) as determined by the DESeq2 analysis.

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CHAPTER 4

INTERNAL FRUIT ATMOSPHERE

INTRODUCTION

Fruit photosynthesis may not only contribute to carbohydrate acquisition by the organ, but may also function in the refixation of carbon dioxide (CO_2), and may release oxygen (O_2) within internal fruit tissues which may aid in the mitochondrial electron transport chain (mETC). Fruits respire at very high rates during early fruit growth to provide energy for the translocation of sugars for growth (Ono et al., 2022). This could lead to internal tissues that experience a hypoxic or even completely anoxic environment as oxygen is used up as the final electron acceptor of the mETC (Noguchi & Yoshida, 2008). Low oxygen conditions can occur for a variety of reasons and the presence of oxygen can be observed as a part of developmentally programmed checkpoints, or in response to stress conditions (Weits et al., 2021). Detection of oxygen is such a critical ability that both plants and animals have very specific, and sometimes analogous, oxygen-sensing pathways as a way to adapt to the sudden increase in oxygen in the Earth's atmosphere 360-300 million years ago (Holdsworth & Gibbs, 2020). In *Arabidopsis,* plants activate 49 core hypoxia-related genes in all tissues once a state of hypoxia has been detected (Loreti & Perata, 2020). Sudden hypoxic conditions generally occur during flooding when the soil is waterlogged, with less than 10% of the soil volume containing air spaces (Salvatierra et al., 2020). In contrast to environmental hypoxia, developmental hypoxia is

evident in seeds and other structures as part of the natural growth processes of organs and is not considered a stressful event for the plant. For example, the meristems of *Arabidopsis* were found to benefit from hypoxic environments and the developmental program of organ differentiation was slowed when additional oxygen was introduced to these tissues (Weits et al., 2019). In any of these situations, plants have developed ways to handle anaerobic conditions through metabolic and physiological acclimation.

Postharvest storage conditions can be hypoxic and are carefully monitored environments used to reduce the metabolic activity of fruits to prevent further ripening. Apples can be stored from a few months up to a year in appropriate conditions, where oxygen levels are typically between 1-3 kPa (Cukrov et al., 2016). In these conditions of oxygen deficiency, the mitochondrial electron transport chain can quickly run out of final electron acceptors and switch to a fermentative metabolic program that requires starch hydrolysis to fuel the production of energy molecules, leading to the production of lactic acid or ethanol after the appropriate pH change of the cytoplasm (Benkeblia, 2021). Extremely low oxygen concentrations can put apples in storage at risk for developing fermentation disorders, and in some cases a dynamic controlled atmosphere storage approach is recommended once apples have reached a critical level of ethanol production so that oxygen levels do not fall below the lower O_2 limit (LOL) and induce disorders (Weber et al., 2015). Reoxygenation can sometimes be dangerous as well, with cell damage and chlorophyll catabolism altering metabolism and physiological processes after flooding events (León et al., 2021). Short-term reoxygenation responses of apple fruit can include dramatic shifts in fermentative pathways, changes in hormonal signaling, and a redirection of propanoid synthesis (Brizzolara et al., 2019). Alanine

accumulation may occur to prevent excess pyruvate from building up in tissues and enhancing fermentation in a harmful metabolic cycle (Fait et al., 2008). However, the GABA shunt may also play a role in reducing the acidification of the cytoplasm by rerouting substrates from fermentation to incorporation into GABA and alanine (Boeckx et al., 2019).

Physiologically, plants have several ways of combatting hypoxic scenarios. One of them consists of expanding the porosity of the tissue. Increasing porosity of tissues allow cells access to more gaseous spaces through which O_2 can diffuse. The formation of extensive air spaces is often achieved via aerenchyma formation (Visser & Bögemann, 2003). Aerenchyma are large air spaces within tissues and are used to facilitate gas exchange. In addition to providing more access to air, the development of aerenchyma could lead to the alleviation of built-up gases so as to release internal tissues of pressures associated with increased gaseous compounds such as ethylene (Loreti & Striker, 2020). Two types of aerenchyma formation have been noted in plants– schizogenous and lysigenous. Schizogenous aerenchyma are void spaces that develop because of programmed cell separation within the plant tissue (Evans, 2004). These generally develop as a part of developmental hypoxia where the low oxygen conditions are not seen as stressful to the plant and instead act as a trigger to begin rearrangement of tissues. Roots of many aquatic plants develop schizogenous aerenchyma, and an indicator of that can sometimes be a visually-organized pattern of gaseous spaces. In contrast, void spaces that arise from lysigenous origins do not seem evenly distributed or as part of a pattern. Lysigenous aerenchyma may develop from sudden stressful events like flooding where void spaces are caused from cells lysing and not from programmed separation. The sudden death of cells can potentially be a result of the buildup of ethylene (Hartman et al., 2021). In apple fruit, it is

theorized that the gas spaces found within internal tissues are of lysigenous origin (Verboven et al., 2008). Even with functional stomata in the early phases of fruit development and lenticel development during later phases of growth, gas diffusion deep into the inner fruit tissues becomes increasingly ineffective due to bulky cortex tissue. Despite lenticels being sites of gas and water exchange in mature fruit, it acts most significantly as a conduit for increased water loss and provides little air flow through the cuticle (Khanal et al., 2020). Within flat structures such as leaves, gas must diffuse through boundary layer resistances, stomatal resistance, and intercellular airspaces before being able to dissolving into a liquid state (Harrison et al., 2020). It is proposed that the air spaces in apples are due to lysigenous origins that begin and end during the cell division stage of fruit growth, whereas additional formation of void spaces is more likely to be the cause of schizogenous aerenchyma whereby the middle lamella separates during latter phases of growth (Herremans, Verboven, Hertog, et al., 2015).

X-ray tomography analyses indicated that the void spaces in apple fruit were always larger than in pear, with the void fraction consisting of about 23% in apple compared to only 5.1% in pear (Verboven et al., 2008). This is relatively consistent with other reports of the internal porosity of fruits such as apples at 23.8-25%, pears at 1.7%, peaches at 2.6%, and mangoes at 9.9% (Cukrov, 2018). X-ray micro-CT results of another study indicate differences in porosity of 'Braeburn,' 'Kanzi,' 'Jonagold,' and 'Conference' cultivars of approximately 18.4%, 12,1%, 25.4%, and 5.7% with oxygen diffusion coefficients of 1.73, 2.73, 10.1, and 0.28 10^{-9} m^2 s^{-1} with all measurements being performed after fruits were harvested in September and October at maturity (Herremans, Verboven, Verlinden, et al., 2015). In similar studies, the cortex tissue always had a higher porosity than the pith, and the porosity of pith tissues did not

change over the course of fruit development, with an average pith porosity of 14.5% in 'Jonagold' fruit (Herremans, Verboven, Hertog, et al., 2015). However, these parameters of internal air spaces are complex, and do not necessarily directly relate to the ease with which gases can traverse void spaces. Herremans, Verboven, and Hertog (2015) also found that porosity and surface area of void spaces played a bigger role in contributing to diffusion as internal spaces were very disconnected. In apple, the void spaces were found to have more surface area, yet the air channels do not themselves connect frequently as in pear, which has effects on the transportation of respiratory gases in the fruit organ.

These air spaces could develop from hypoxic conditions that exist early in fruit development partially due to high rates of respiration. In a study done on 'Braeburn' apples, researchers found that there was a decline in the amount of air inside the fruit tissue going from the outer parts of the fruit (0-3 mm inside the fruit) to the center pith (27-30 mm inside the fruit), with the outer portions having up to 20-25% air space and the inner portions only having 5-10% air space (Drazeta, 2004).

Altogether, the respiration rates of early fruit growth could be influencing the development of air spaces within the fruit. This increase in air space could alleviate hypoxic conditions that exist deep within the fruit where tissues are already constrained by physiological parameters. Examining internal oxygen concentration within the fruit and their relationship with fruit respiration can provide a better understanding how internal atmosphere of fruit changes during early fruit development. It is hypothesized that chronic hypoxia occurs during early fruit development and that it is associated with high fruit respiration. To test this hypothesis, fruit

internal O2 concentrations and respiration rates were measured during multiple stages of early fruit development in two apple cultivars.

MATERIALS AND METHODS

Internal O_2 concentration

Internal O_2 measurements of 'Golden Delicious' and 'Fuji' fruits were taken with a Presens optical O_2 sensor (Presens, Germany). The sensor was calibrated using a two-point calibration water saturated with O_2 (air) and with water depleted of O_2 using sodium sulfite and cobalt as a catalyst, as per the manufacturer's instructions. Measurements were taken at 15, 22, 29, 36, 43, 50, and 71 d after full bloom (DAFB). Fruit were collected, brough to the inserted with the Presens probe that contained the optical sensor. The probe was inserted 5-12 mm into the fruit cortex to determine the internal concentration of oxygen. This region corresponded to the inner cortex of the developing fruit. The sensor was left in for up to two minutes and only stable measurements after approximately 20 seconds of insertion were used for analyses.

Respiration measurements

Measurements of 'Golden Delicious' and 'Fuji' fruit respiration were performed in two different ways. For all fruit, after collection the fruit were stored at 4C in the dark. The fruit were collected 36 and 43 DAFB and were brought back to room temperature before measurements began and before obtaining cores for the respiration experiments.

The closed chamber method for respiration measurements was performed using a mason jar to measure increased carbon dioxide evolution. This was performed at several stages

of fruit development. Additionally, the Presens optical sensor was used to examine O_2 consumption from dissected fruit tissues. For the former method, mason jars were aired out with fresh air and had varying numbers of apple fruit (from 4-10 depending on the size) placed into a jar covered with aluminum foil and sealed with tops that had rubber stoppers in them. 'Golden Delicious' fruit weight for 15, 22, 29, 36, 43, 50, and 71 DAFB were 2.75 ± 0.37, 6.2 ± 1.192, 9.625 ± 1.825, 25.2 ± 5.611, 38.175 ± 6.66, 59.5 ± 11.654, and 122.55 ± 6.031 mg. 'Fuji' fruit weight for 15, 22, 29, 36, 43, 50, and 71 DAFB were 2.5 ± 0.432, 2.575 ± 0.486, 7.7 ± 0.535, 22.025 ± 2.487, 32.1 ± 4.927, 48.575 ± 6.353, and 94.525 ± 6.326 mg. Control jars did not contain any fruit yet were also flushed with air and capped in a similar manner. After one hour of incubation in the dark, a 60 mL syringe was inserted through a rubber septum in the lid of the jar and used to thoroughly mix the air within the mason jar. Then, 60 mL of air was drawn out from the mason jar and slowly injected into a CO_2 analyzer (Quantek Instruments, USA). The percent of carbon dioxide in the headspace was used in conjunction with the jar headspace volume, fruit weight to calculate the concentration of carbon dioxide in µmol g^{-1} h^{-1} at room temperature.

The second method to measure respiration was performed using the Presens optical sensor to measure the amount of O_2 consumed by fruit tissue immersed in a buffer prepared with 100% saturated distilled water. The measurements were performed once 36 DAFB and once 43 DAFB for both cultivars. To saturate the water with oxygen, a small tube that expelled air was inserted into a 50 mL centrifuge tube of dH_2O for approximately 20 minutes. After fully saturating the water with air, the water was used to make a buffer containing 100 µL MES, 100 µL HEPES, and 400 µL $CaCl_2$ which was then made up to 10 mL with air-saturated water. Then,

1 mL of buffer was dispensed inside amber vials containing cores of apple fruit tissues. The apple cores were taken from two areas of the cortex: the inner and the outer. Distinguishing between the inner and outer cortex was done by determining the distance between the peel and the vascular bundles and then estimating the approximate location of the two spatial areas. These cores were slightly dried using kimwipes to remove surface sap and were inserted into the amber vials and then crimp-sealed with an airtight GC cap with a rubber septum. Measurements of the O_2 concentrations in the vials at 0, 20, 50, and 80 min after incubation were obtained using the needle probe of the Presens optical sensor to allow for tracking of internal tissue respiration *via* O_2 consumption. Oxygen concentration data over time were plotted to determine the rate of respiration and expressed as the rate of O_2 consumption.

RESULTS

Internal O_2 concentration

The percent oxygen within fruits exhibited markedly different patterns, with 'Golden Delicious' fruit displaying an increase in O_2 concentration over time, while 'Fuji' fruit remained at relatively low levels throughout the duration of the experiment, from 22 DAFB to 71 DAFB. Both cultivars showed very low levels of internal O_2 during early fruit development indicating severe hypoxia. 'Golden Delicious' fruit O_2 concentrations averaged at 0.166 ± 0.025% oxygen within the tissue at 22 DAFB, and 'Fuji' at 0.778 ± 0.571% at the same date. Over time, the internal oxygen percent of 'Golden Delicious' steadily increased to 8.03 ± 1.6% at 71 DAFB while 'Fuji' remained relatively stable at 0.929 ± 0.44% at 71 DAFB.

Respiration measurements

The results from the CO_2 evolution experiment using the jars showed a clear decline in the respiration rate from 15 DAFB to 71 DAFB. 15 DAFB measurements of 'Golden Delicious' and 'Fuji' show an average of 10.7 ± 0.515 and 11.0 ± 0.75 μmol of CO2 being produced per gram of tissue per hour, respectively. For 'Fuji', respiration rates increased for the 22 DAFB measurement, with an average value of 16.8 μmol /g/h before declining for 29, 36, 43, 50, and 70 DAFB with values of 6.51 ± 0.311, 5.73 ± 0.0244, 4.1 ± 0.275, 3.35 ± 0.16, and 1.64 ± 0.0277 μmol /g/h, respectively. The 'Golden Delicious' values exhibited a steady decline throughout the experiment with values of 7.38 ± 1.2, 6.3 ± 0.179, 5.01 ± 0.313, 4.17 ± 0.221, 3.27 ±0.0459, and 1.84 ±0.0831 μmol /g/h for 22, 29, 36, 43, 50, and 70 DAFB respectively. There was no statistical difference between respiration measurements of the cultivars except for the 22 DAFB measurements. The 'Fuji' fruit displayed statistically higher respiration rate than the 'Golden Delicious' fruit for that one measurement only. 'Fuji' replicates 1, 2, 3, and 4 had Log_2fold increases of 1.78, 0.97, 0.583, and 1.465, respectively when compared to the 'Golden Delicious' rates.

Respiration measurements using the Presens O_2 sensor showed a decline in the concentration of oxygen in the buffer over the course of 80 min, as expected. No tissue-level significant differences were noted between the inner and outer cortex tissues for any date. Measurements 36 DAFB, 'Golden Delicious' samples began with 8.71 ± 0.0303 ppm oxygen and ended the 80-minute experiment with 6.23 ± 0.369 ppm oxygen. Similarly, 36 DAFB 'Fuji' samples had 8.75 ± 0.02 ppm oxygen at minute 0 and 6.73 ± 0.236 ppm at minute 80. For 43 DAFB, samples began at approximately 8.62 ± 0.0225 ppm for 'Fuji' and 8.72 ± 0.02 ppm for

‘Golden Delicious’ at minute 0 before declining to 6.86 ± 0.272 ppm for ‘Fuji’ and 6.57 ± 0.275 ppm for ‘Golden Delicious’. The respiration measurements for the inner and outer cortex did not statistically vary from 36 to 43 DAFB. These measurements of oxygen were then used to calculate rates of respiration by taking into consideration the fruit weight. The rates were largely similar as well, with no statistical difference between either the tissue or the cultivar. Because there were no tissue-level differences, ‘Fuji’ fruits as a whole had a rate of 2.1 ± 0.124 nmol/g/h 43 DAFB. ‘Golden Delicious’ fruits had rates of 1.93 ± 0.076 nmol/g/h 43 DAFB.

DISCUSSION

Cultivar differences were apparent in regards to the internal atmosphere of fruits, since we can see differences in the way that ‘Fuji’ and ‘Golden Delicious’ responded in many of the experiments. This could be because the network of gas spaces within apple cortexes of different cultivars are not the same, and a given apple cultivar may be physiologically less conducive to gas transport than others. Consistently, ‘Kanzi’ apple varieties were reported to have a pore space of 12.1% ± 3.0%, while ‘Jonagold’ apples contained 25.4% ± 2.6% pore space. This is also in contrast to another pome such as the ‘Conference’ pear that has pore spaces of only 5.7% ± 1.4%. (Herremans, Verboven, Verlinden, et al., 2015). Pore spaces within each variety have a different level of connectedness that could make gas transport pathways more tortuous and influence how well oxygen or carbon dioxide moves within tissues.

The results from the CO_2 evolution measurements clearly displayed declining respiration from 15-71 DAFB. These data verify that there is initially a high level of respiration occurring which then steadily decreases. Initially, the amount of oxygen within the fruit tissues was very

low and internal fruit tissues were extremely hypoxic. The presence of oxygen increased throughout the early development of the apple fruit, but according to observed results, this was evident only in 'Golden Delicious'. Hence, in this cultivar, it is possible that decrease in tissue respiration potentially coupled with increased tissue porosity, alleviated hypoxia within internal fruit tissues. However, although respiration decreased similarly during early fruit development in 'Fuji', no alleviation of chronic hypoxia was noted, suggesting that these two processes may at least be partially uncoupled. Potentially, lack of rapid establishment of void spaces within the cortex contributes to the lack of hypoxia alleviation in 'Fuji'. Analyses of pore space development in the cultivars is likely to provide more information regarding the alleviation of chronic hypoxia during fruit development.

Although some fruits could perform photosynthesis to alleviate the hypoxic conditions, it seems unlikely that it is contributing significantly to the prevention of hypoxia in apple since some fruits have different spatial arrangements of cells that seem to be ineffective for gas transport. High levels of disconnected air spaces may make transport of gases so inefficient that it potentially reduces the effectiveness of fruit photosynthesis in alleviating hypoxia within internal fruit tissues.

Additionally, the oxygen measurement data using the Presens sensor tracked the steady decline in oxygen availability within the saturated water solution. There was not a difference in the respiration levels of inner and outer cortex samples, as determined from the results. Together, these results demonstrate that cortex of apple fruit respire at relatively the similar rates regardless of their internal position in the fruit, at least during the early stages of fruit development. Because the cortex composes the vast majority of apple fruit tissue, it is

reasonable to assume that the entire cortex is respiring at statistically similar rates. The interal tissue respiration measurements indicate potential respiration capacity as they were performed under conditions where tissues were incubated in saturated O_2 concentrations. The relative amount of O_2 available within deeper parts of the cortex owing potentially to the greater tortuous path for diffusion, and the lack of void connectivity may still alter the O_2 concentration within inner tissues thereby reducing their effective respiration. Because of the already-present gradient of oxygen in the core tissues of apple fruit, the inner parts of the fruit are exposed to considerably more strain in terms of oxygen availability than the more outward parts of the fruit.

CONCLUSION

This study presents evidence for chronic hypoxia during early fruit development in apple through direct measurements of oxygen in the fruit. High rate of fruit respiration via mETC during these stages of fruit development likely contributes to chronic hypoxia development. Chronic hypoxia was relieved only in 'Golden Delicious' at later stages of development, but not in 'Fuji' although fruit respiration declined similarly in both cultivars. Hence, porosity and connectivity of internal spaces is also likely to influence internal O_2 concentrations

FIGURES

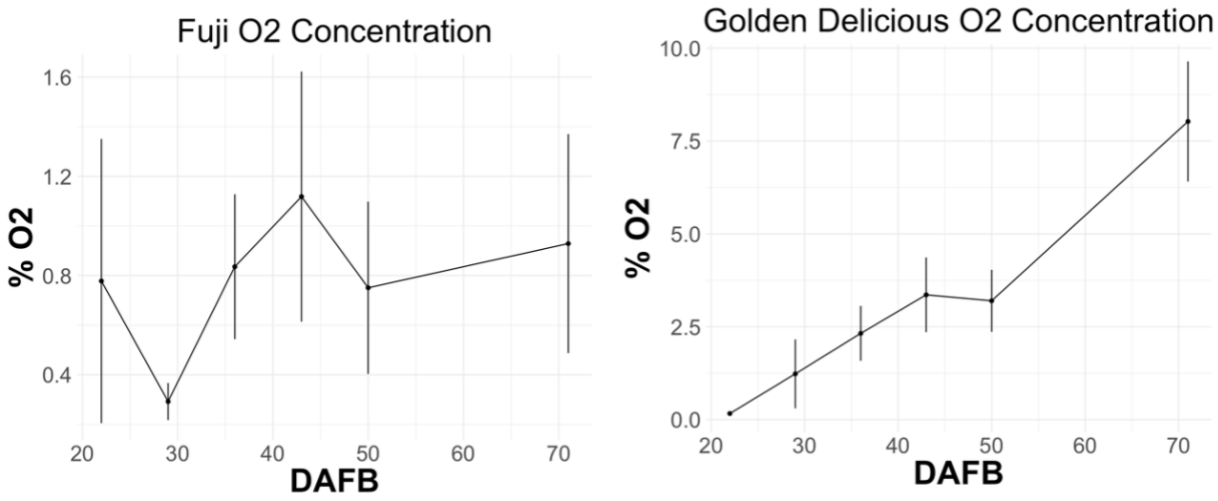

Figure 18. Internal oxygen concentration in the apple fruit during early and mid-fruit development at 15, 22, 29, 36, 43, 50, and 71 DAFB. Oxygen concentration was measured in the internal fruit cortex tissue in 'Fuji' and 'Golden Delicious' apple using a PreSens optical sensor. The mean ± SE O_2 concentration are presented.

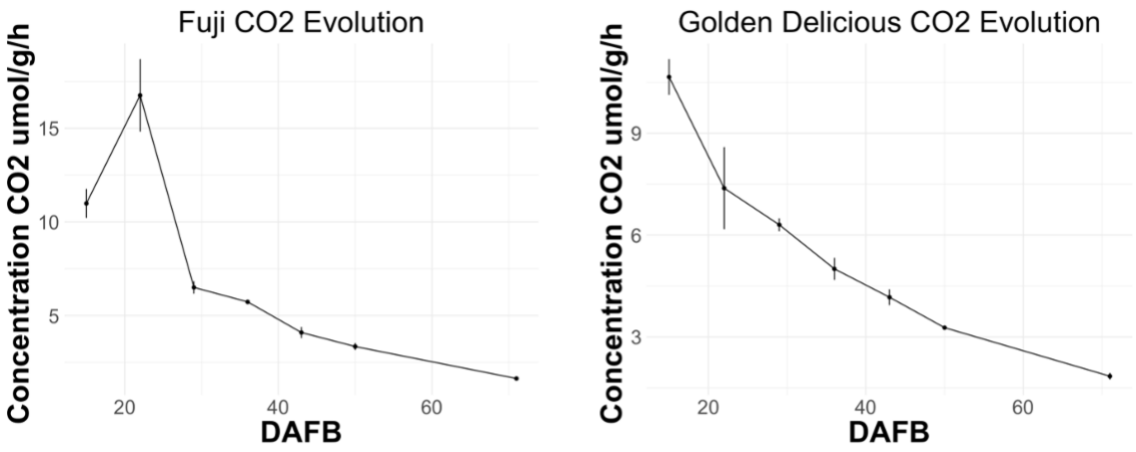

Figure 19. Carbon dioxide evolution from the apple fruit during early and mid-fruit development at 15, 22, 29, 36, 43, 50, and 71 DAFB. Carbon dioxide concentration was measured in 'Fuji' and 'Golden Delicious' apple by incubating fruit in jars for 1 h and then injecting 60 mL of headspace gas into a CO_2 analyzer. The mean ± SE O_2 concentration are presented.

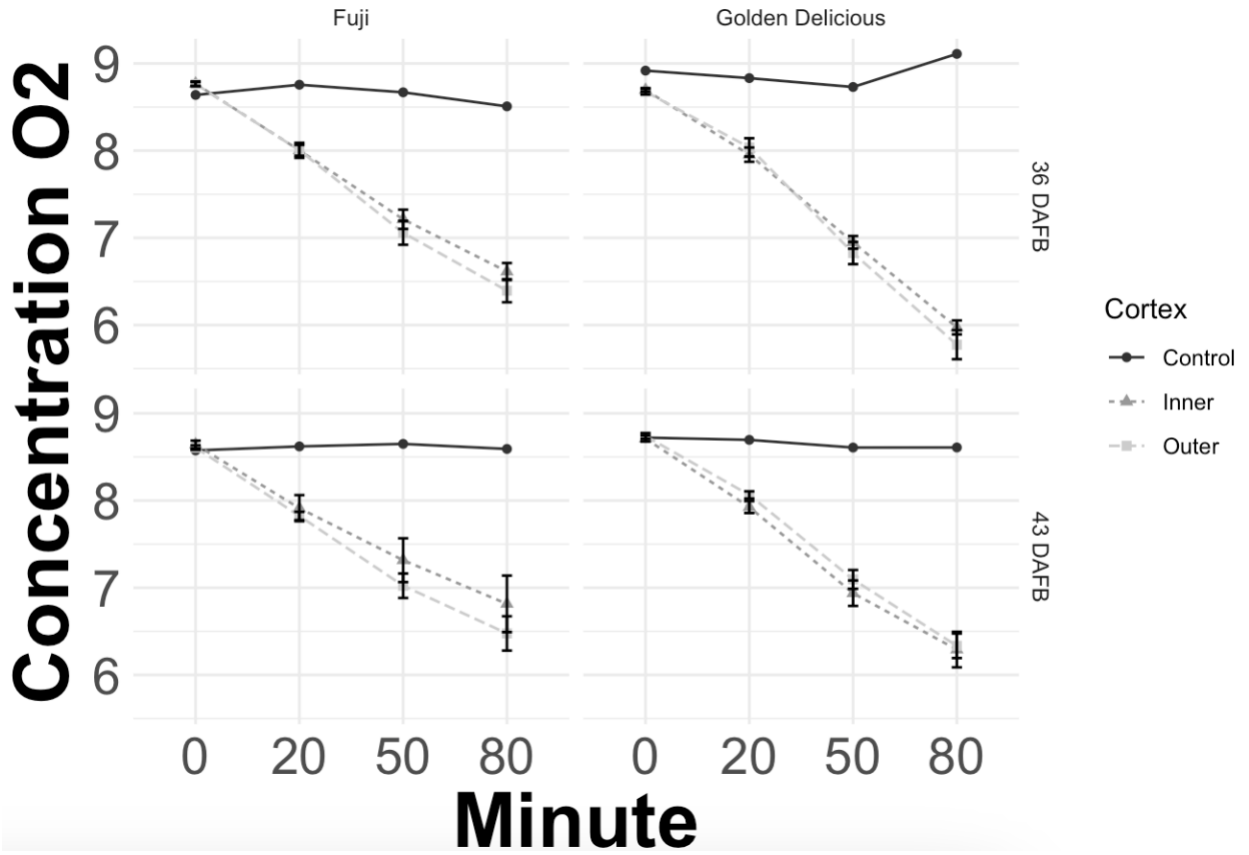

Figure 20. Declining concentrations of oxygen in vials with apple fruit tissues in a buffer saturated with 100% oxygen during mid fruit development (36 and 43 DAFB). Cores of 'Fuji' and 'Golden Delicious' cortex tissue (inner or outer) were placed in the vials and incubated in the dark at room temperature. The mean ± SE O_2 concentration are presented with the control as the reference. There was no significance when comparing treatments or cultivars.

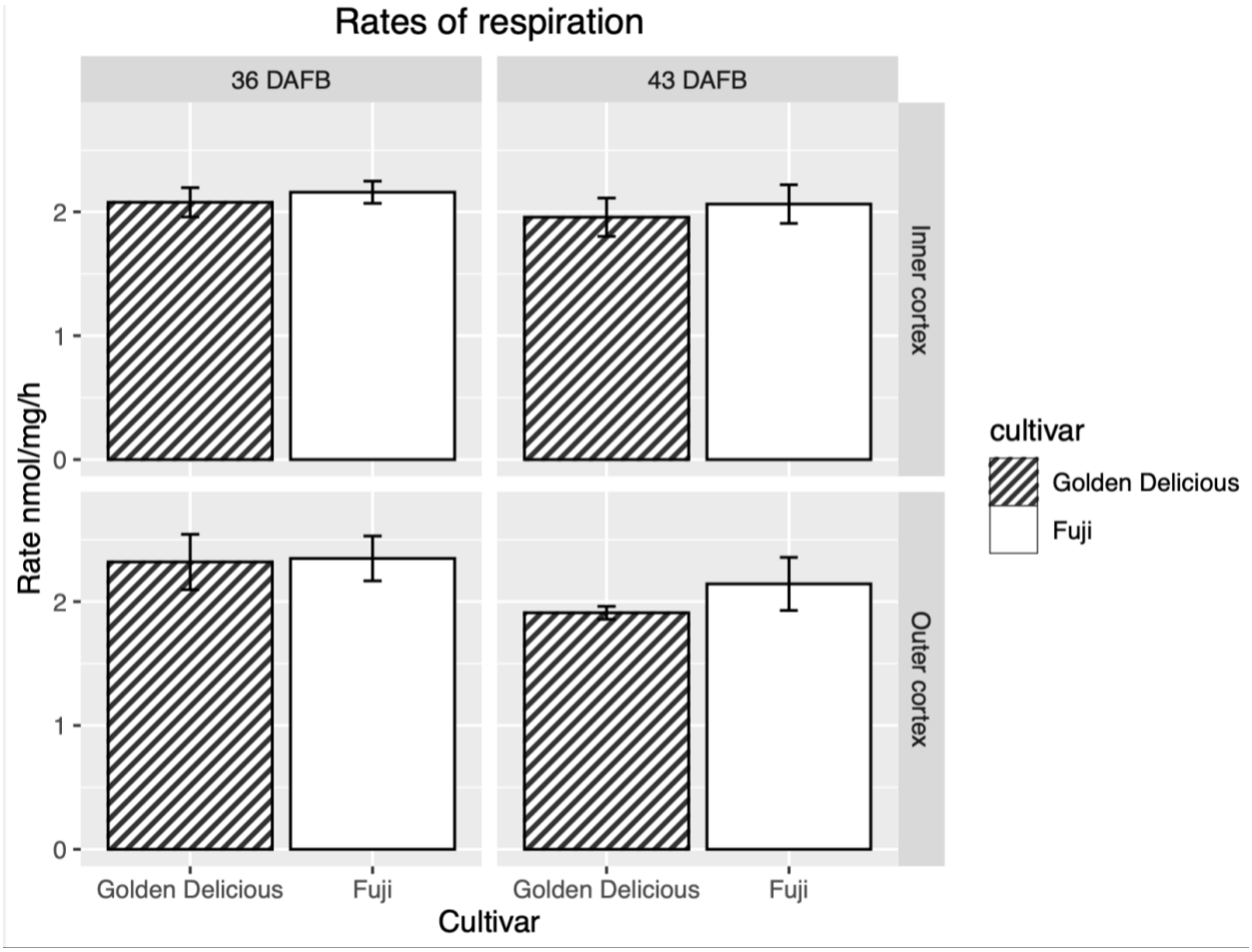

Figure 21. Rates of respiration in the apple fruit during mid-fruit development (36 and 43 DAFB). A Presens optical sensor was used to measure oxygen concentration in a vial (Figure 20) and rates of respiration were calculated for each cultivar with these values. The mean ± SE O_2 concentration are presented. There was no significance when comparing treatments or cultivars.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The overall goal of this study was to understand early fruit growth and development in apple. These investigations ranged in purpose from understanding the contribution of fruit photosynthesis to growth and development, to genetic factors that contribute to high growth rates, and the development of chronic hypoxia in the fruit. The results of the fruit photosynthesis experiments revealed that fruit photosynthesis influences carbohydrate metabolism, plastid ultrastructure, metabolite concentration, starch concentration, and gene expression. Results from the growth rate study helped identify that processes such as nucleosome assembly, cell division, and chromatin assembly are critical to sustain high rates of fruit growth. Further, evidence for the development of severe and chronic hypoxia was found within the developing apple fruit. Hypoxia was alleviated only partially and inconsistently by decrease in respiration rates at later stages.

Future experiments could be performed to continue to reveal the ways in which the apple fruit grows and develops during the first 30 DAFB. Next steps for the photosynthesis experiments could be performing RUBISCO activity assays as well as qPCR on various genes like *RUBISCO* and *PEP CARBOXYLASE* to reveal the extent to which fruit may photosynthesize and how it compares to foliar photosynthesis. While these were attempted in the current study, further optimization is needed to successfully use the results in future studies. The growth rate

study could be enhanced by collecting a larger volume of fruit and then taking care to have enough tissue to perform RNA extractions, as well as metabolite and phytohormone analyses. A large bottleneck to this experiment was the lack of available tissue quantities and the subsequent pooling of material that had to be performed. More sensitive extraction techniques in the future may allow for quantification of these variables from individual fruit. Such data may help identify growth related processes in greater detail. Additionally, performing micro-CT scans on our apple cultivars, and or performing internal atmosphere experiments on apple cultivars that have X-ray scans already performed could advance our knowledge on the third objective of this study. These tools are likely to provide better information regarding pore space development within the fruit. By comparing fruits that have different levels of porosity the contributions of respiration rates and tissue porosity to hypoxia development may be ascertained.