THESIS

BRIDGING THE GAP BETWEEN BIOFORTIFICATION AND CONSUMPTION: EVALUATING SORGHUM GRAIN CAROTENOID DEGRADATION

Submitted by

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ABSTRACT

BRIDGING THE GAP BETWEEN BIOFORTIFICATION AND CONSUMPTION: EVALUATING SORGHUM GRAIN CAROTENOID DEGRADATION

Sorghum (*Sorghum bicolor*) is a major staple cereal crop consumed in sub-Saharan Africa and Southeast Asia, where some of the highest rates of vitamin A deficiency (VAD) are found. As with most cereals, sorghum has low concentrations of provitamin A carotenoids, which are converted to vitamin A in the body. Biofortification provides an opportunity to address VAD through the nutritional improvement of sorghum grain using a non-transgenic breeding approach to increase grain carotenoids. Though vitamin A biofortification in sorghum is possible, it is unknown if breeding for high carotenoids in the grain negatively affects carotenoid pathway functions in other tissues. Additionally, it is unknown if degradation during postharvest processing occurs to a significant degree in biofortified grain. To establish how breeding for high carotenoids in the grain affects the carotenoid pathway in other plant tissues, expression of ten genes in the carotenoid precursor, biosynthesis, or degradation pathways were evaluated in the grain, leaf, and root tissues. A correlation in the gene expression within the plant tissue, but not between the plant tissues, was found for most genes, which suggests that several of the carotenoid precursor, biosynthesis, and degradation genes are controlled by tissue-specific regulation. Correlation of carotenoid concentrations and gene expression was also found to be tissue specific, which further suggests tissue-specific regulation. The selection of genes with tissue-specific regulation for marker-assisted breeding reduces the chances of grain biofortification negatively affecting other tissues. Once carotenoids have been increased in the

grain, it must be noted that vitamin A is not stable in most storage, processing, and cooking environments due to oxidative stress from light, heat, and oxygen. The degradation of the nutritional quality through post-harvest processing was evaluated by sampling carotenoid grain throughout harvest, drying, storage, processing, and cooking. Individual processing steps did not cause significant degradation but added up to significant degradation by the final cooking step, with ~39% of β-carotene loss. No significant difference between the loss in the different storage temperatures or cooking styles was seen. An increase in the target value from 4 μg β-carotene/g of sorghum to 5.6 μg/g will be needed to account for processing loss in order to provide 50% of the estimated average requirement (EAR) of vitamin A. Overall, both the information on tissue specific gene expression, and post-harvest degradation will further advance the development of carotenoid biofortified sorghum lines.

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Chapter 1: Reducing Malnutrition Through Sorghum Carotenoid Biofortification

1. Introduction to the Problem and a Potential Solution

After decades of decline in global hunger, it is on the rise again as the world faces new challenges. Climate change, global conflicts, and the COVID-19 pandemic have disrupted established practices of farming, processing, and distribution of staple crops, leading to reduced access to food (FAO, IFAD, UNICEF, WFP, & WHO, 2021, Yu & Tian, L. 2018). Decreased access to nutritious food leads to malnutrition, which increases the incidence of morbidity and mortality, and globally contributes to 45% of deaths in children under five (World Health Organization, 2021). Vitamins and minerals are needed to produce metabolites that are essential for growth and development, so deficiencies in essential micronutrients have serious negative health effects and currently affect 1 in 3 people globally (International Food Policy Research Institute, 2014). Areas primarily affected by micronutrient deficiencies include sub-Saharan Africa and Southeast Asia, where high rates of iodine, iron, and vitamin A deficiencies impact the population (World Health Organization, 2021). Deficiencies in these nutrients can lead to a range of health concerns, such as impaired immune function, increased risk of infections, and increased risk of chronic diseases such as cancer and cardiovascular disease (Shenkin, 2006). While current solutions such as supplementation programs have shown some progress in improving nutrition status, additional solutions are needed to aid in the prevention and treatment of micronutrient deficiencies.

The Green Revolution brought extensive advancements in plant breeding and led to the development of elite commercial crops, providing farmers with high yields that quadrupled the production of staple cereals from 1961 to 2014 (Ritchie, 2017). However, while genetic advances

in crop improvement provided more food, there was no parallel increase in crop nutritional quality. Current breeding efforts aim to incorporate nutritional improvement strategies into elite variety development, but in practice, this has not been carried out extensively due to the complexities of incorporating nutritional traits into breeding pipelines, as well as regulatory hurdles for biotechnology approaches. This untapped potential in improving crop nutritional quality provides an opportunity to alleviate global malnutrition through biofortification breeding of staple crops.

1.1 Vitamin A Deficiency

Vitamin A deficiency (VAD) decreases immune function, resulting in an increased risk of infections and death from common illnesses; increases maternal blindness and maternal mortality; slows growth and development; and is the leading cause of preventable blindness in children under five years of age. An estimated 30% of children under five suffer from vitamin A deficiency, which accounts for 2% of their deaths (Wirth et al., 2017). Considered a public health crisis in almost half of the world's countries, vitamin A deficiency also negatively impacts the global economy. Chronic diseases caused by VAD leave workers unable to perform their essential duties, and increased child mortality decreases the influx of new workers. With the physical, mental, and economic consequences, VAD is seen as the second biggest risk factor to the global disease burden that is directly linked to the lifespan of affected populations (Zhao et al., 2022).

Vitamin A is an essential micronutrient that cannot be synthesized by the human body, so it must be obtained through diet. In many populations, the majority of vitamin A is obtained through consumption of carotenoids, which can be found in a multitude of plant foods, including

yellow, orange, and red fruits, and leafy greens. Provitamin A carotenoids (pVAC) are those that are converted to retinol (vitamin A) in the body, and β-carotene is one of the most abundant provitamin A carotenoids found in plants. In addition to plant carotenoids, the other source of vitamin A in the human diet is preformed retinol in animal products (Hodge & Taylor, 2023).

The estimated average requirement (EAR) is the average daily amount of a particular nutrient that is required for half the healthy individuals in a population. The proportion of the population with an intake below the EAR is the target population for nutritional improvement strategies. The EAR for vitamin A is reported in retinol activity equivalents (RAE), which includes both preformed retinol and provitamin A carotenoids. For children 1-8 years of age, the EAR ranges from 210-275 μg RAE, for children and adults 9 years of age and older the EAR ranges from 4420-625 μg RAE, and the highest EAR is for pregnant and lactating adults with an EAR of 530-900 μg RAE (National Institutes of Health, 2022). The primary pVAC found in food is β-carotene, which can be cleaved into two retinols after consumption, but various factors, including the food matrix of plant foods, can reduce carotenoid bio accessibility, so the conversion efficiency needs to be considered when estimating the amount of β-carotene that is converted into retinol. A ratio of 12 β-carotene to 1 retinol is commonly used, but the ratio varies considerably depending on several factors, including physical factors in the edible plant part and how the plant is prepared into food (Pfeiffer, 2013, Tang, 2010).

There are several complementary methods to improve nutritional status, including dietary diversification, supplementation, fortification, and biofortification. Dietary diversification, wherein carotenoid-rich foods that were not previously consumed are introduced into the diet, is the ideal intervention, but is not possible for many populations where there is little access to growing or purchasing diverse foods. Supplementation and fortification programs have been

successful in many regions of the world, and many countries have supplementation programs that dispense high doses of preformed vitamin A, primarily targeting young children. However, continuous supplementation (every 3 months) and financial support are needed, and the programs often do not reach the neediest populations in remote rural areas.

1.2 Biofortification

Biofortification is a technique used to nutritionally enhance a food crop through genetic engineering or plant breeding. Unlike fortification, which adds nutrients through processing, this technique increases nutrients during the plant's growth (Bouis et al., 2011). While genetic engineering of cereal crops has been successful, they have been met with major regulation and policy obstacles in countries needing them the most (Khush et al., 2012). The introduction of genetically engineered crops to food insecure regions has often been met with resistance as the products have not been designed to fit into the culture or policies of the area, making them ineffective solutions (Diepenbrock & Gore, 2015). Cultural sensitivity and community involvement in nutritional improvement efforts are exceedingly important for the success of the programs.

Since genetically modified foods have not yet gained the trust of consumers, traditional breeding remains the most viable approach to biofortification. Biofortification breeding provides an effective long-term solution for nutritional improvement, as it develops nutritionally enhanced varieties that the target populations already know how to cultivate and use in cultural dishes. However, a limitation of traditional breeding is the amount of time—typically ten or more years—it takes to breed multiple generations to achieve a significant accumulation of nutrients. Identifying the genetic controls underlying variation in nutrient concentrations can accelerate

traditional breeding by providing genetic tools that can quickly identify progeny in the breeding population that harbors favorable alleles (Diepenbrock & Gore, 2015).

In order to effectively develop biofortified crops through breeding, organizations such as Harvest Plus, land-grant universities, and government agencies have collaborated to develop similar step-by-step scale-up strategies to cover the discovery, development, and implementation of a biofortified product. The potential success of a product is established by evaluation through each of these main steps, but the steps should be thought of as a cyclical rather than linear process, allowing biofortification programs to optimize resources and produce quality products that address all areas of sustainability (Fig. 1).

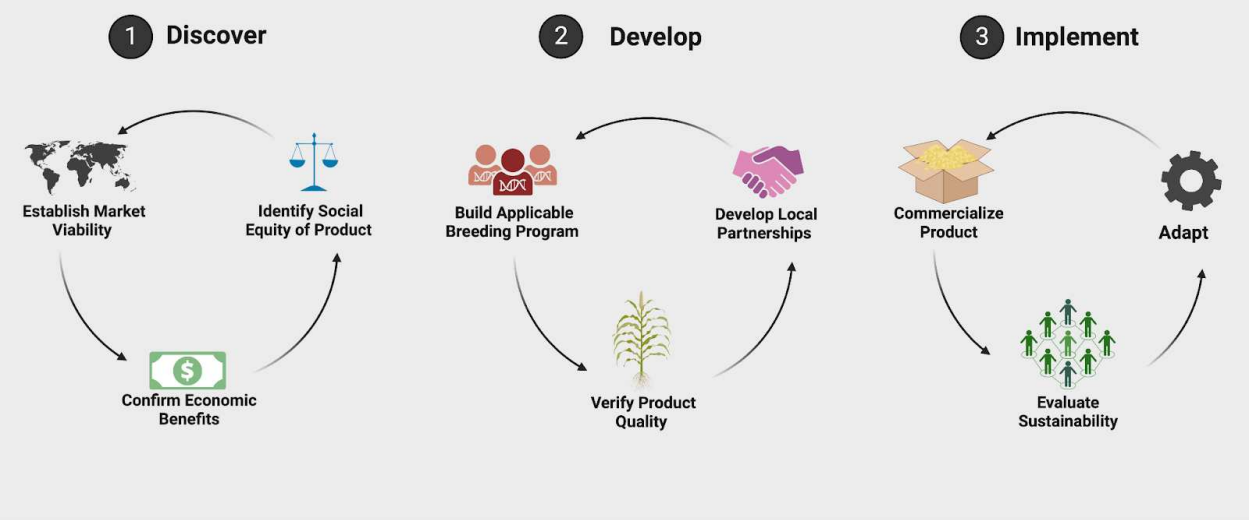

Figure 1.1 Cyclical strategy for sustainable biofortified programs (based on ideas from Harvest Plus, 2022)

The discovery stage consists of identifying the target market, social impact, and economic benefits of a biofortification program. Establishing the market viability of the product will provide evidence for consumer demand and present advantages of biofortified products over other nutrition improvement options. Confirming potential economic benefits—from lowering health care costs and improving productivity to creating opportunities for surplus profit to add to

the local economy—will entice future investments in the product. Most importantly, identifying the social equity of the product by discussing the need for and requirements of a new product with local farmers, producers, and consumers in target communities will ensure the product is useful and benefits as many community groups as possible. This foundational information will allow for a broad assessment of the impact of a biofortified crop and the initial goals for the developmental stage can be created.

The development phase uses the information identified in the discovery phase to design a breeding program, establish local partnerships, and perform quality tests. Applicable scientific research is performed to 1) characterize the natural genetic variation of the nutrient of interest, 2) identify and develop potential genetic tools that can advance traditional breeding, and 3) assess the stability of target nutrients in order to enhance their bioavailability. Production connections are set up to ensure the product can be adequately produced and add value to established practices. An experimental trial can then be used to evaluate the performance of the biofortified product in production, processioning, and consumer environments to compare performance to current commercial varieties. Once goals are met and supply chains are established, the biofortified product can then be implemented in the target market.

In the implementation phase of developing a biofortified product, commercialization, evaluation, and adaptation are needed to ensure the program's sustainability. The success of the new product is significantly influenced by commercial production, processing, and marketing to consumers and can only be accomplished with support and coordination between government entities and community members. It is also important to evaluate environmental, social, and economic shifts to ensure product sustainability for the target market. Lastly, continuous

innovation is needed as current trends, markets, and policies potentially shift the goals of the product.

1.3 Vitamin A Biofortification

Vitamin A biofortification strategies in crops focus on increasing the concentrations of pVAC, which are converted to vitamin A in the body. Carotenoids are yellow, orange, and red-pigmented compounds found in all photosynthetic organisms. Within plants, they aid in light harvesting and photoprotection during photosynthesis, act as antioxidants, and serve as precursors to important plant hormones such as abscisic acid (Vishnevetsky et al., 1999). In humans, dietary carotenoids act as antioxidants and anti-inflammatories, and are associated with protection against chronic diseases and age-related macular degeneration. There are over 600 types of known carotenoids, but only 40 are present in the human diet. The provitamin A carotenoids include α-carotene, β-carotene, and β-cryptoxanthin. The carotenoids lutein, zeaxanthin, and lycopene cannot be converted to vitamin A by humans but can aid in eye protection through blue light absorption (Rao & Rao, 2007).

To identify the best strategy for biofortification breeding, an understanding of each step of biosynthesis and degradation of the compound of interest is needed. The carotenoid pathway has been highly characterized in efforts to understand how regulation at the gene, protein, and metabolite level affects the accumulation of provitamin A carotenoids. Carotenoid production starts with the 5-carbon precursor compounds isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are synthesized through the methylerythritol 4-phosphate (MEP) pathway. IPP and DMAPP are precursors used in multiple pathways to create terpenoids (also called isoprenoids), organic compounds consisting of 5-carbon isoprene units and two or

more hydrocarbons. The condensation of IPP and DMAPP produces geranylgeranyl diphosphate (GGPP), another isoprenoid precursor used in many pathways. Competition between pathways for precursor compounds limits the availability of substrate, creating a bottleneck for downstream carotenoid biosynthesis. Phytoene synthase (PSY) catalyzes the first committed step in the carotenoid pathway, condensing two GGPPs to form phytoene. PSY has been identified in many crop species as the major rate-limiting step in carotenoid biosynthesis and has been highly studied for biofortification efforts (Cazzonelli & Pogson, 2010). At the pathway branchpoint, lycopene β-cyclase (βLCY) has been shown to control flux into either branch, so is another biofortification target.

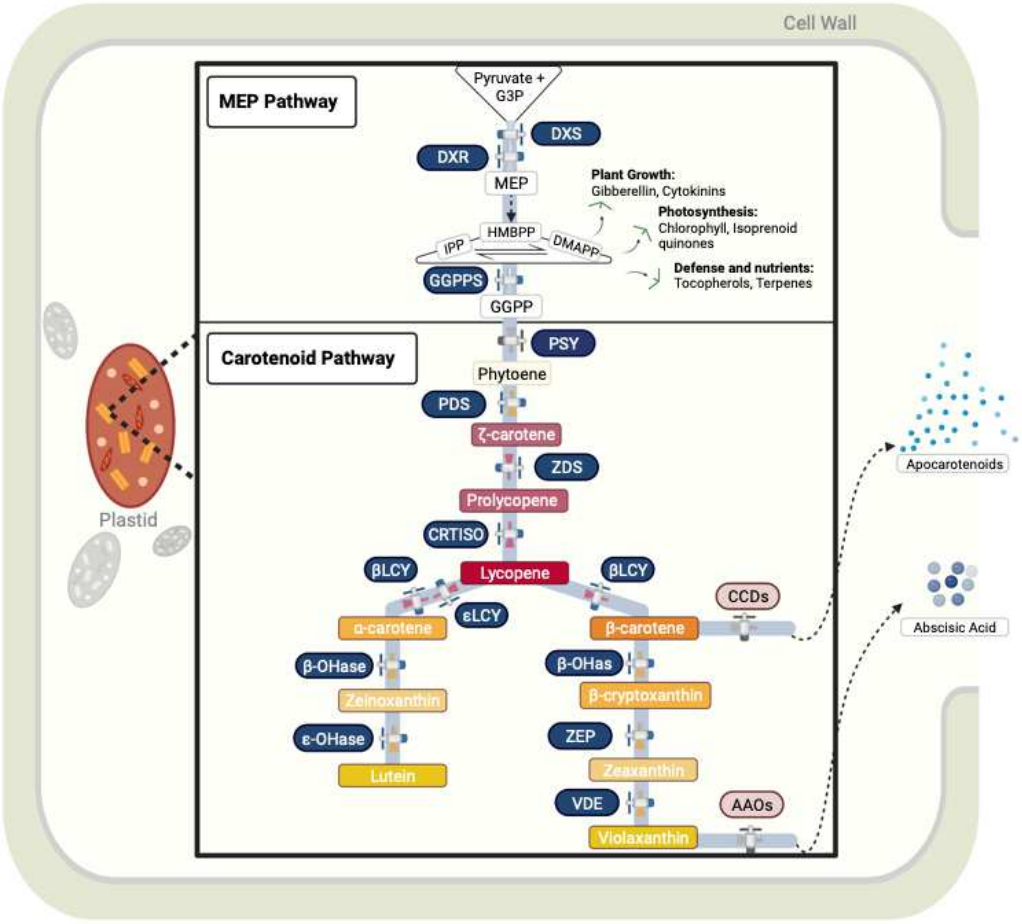

Figure 1.2 Simplified scheme of the MEP and Carotenoid Pathways. Biosynthesis and degradation occur within plastids of plant cells, and degradation products (apocarotenoids) are sequestered to other plant tissues.

Provitamin A degradation genes, including β-carotene hydroxylase (βCH), zeaxanthin epoxidase (ZEP), and ABA aldehyde oxidase (AAO), acting on the β-carotene branch of the pathway are also potential biofortification targets. Additionally, degradation by carotenoid cleavage dioxygenases (CCDs) occurs at several points throughout the pathway, producing a variety of apocarotenoids, such as flavor and aroma compounds and important hormones such as abscisic acid and strigolactone. CCD alleles that slow the rate of carotenoid degradation are another potential target for biofortification breeding. While these are the main genes identified as potentially controlling carotenoid variation, there is a possibility of variation at any point within the pathway.

The identification of rate-limiting steps and degradation points in the pathway provides potential targets for biofortification breeding. Understanding each gene's role in the pathway and how they affect overall carotenoid concentration is the starting point for breeding to increase Vitamin A content (Cazzonelli & Pogson, 2010). Efforts to use traditional breeding, rather than genetic engineering, have been successful in maize and cassava, with biofortified concentrations ranging from 10–15 μg/g of β- carotene and 60 μg/g of total carotenoids (De Moura et al., 2014). Successful biofortification efforts in these two crops provides evidence that similar successes can be achieved in other staple crops.

1.4 Sorghum Carotenoid Biofortification Through Reduction of Degradation

Sorghum (*Sorghum bicolor*) is a major cereal staple crop grown and consumed in sub-Saharan Africa and Southeast Asia, which has some of the highest rates of vitamin A deficiency. Sorghum is a vital crop to farmers in semi-arid regions as it performs better than most other food crops in drought and high-temperature conditions. Sorghum is a multi-use crop, produced for

animal feed, fiber, fuel, and human consumption to provide for millions of people around the world (Kumar et al., 2015). The grain is composed of protein, fiber, and starch, as well as thiamin, riboflavin, niacin, and some phytonutrients. Globally, the human diet consists primarily of staple cereal grains, which provide up to 80% of vitamin A in deficient populations (Awika, 2011). However cereal grains generally have low concentrations of carotenoids compared to fruits and vegetables which are inaccessible to many populations or only available during certain times of the year (Sommer, 2008).

Provitamin A carotenoids in sorghum have recently been studied to explore the possibility of using biofortification breeding to increase the average of <1 μg/g of provitamin A carotenoids to a biologically relevant concentration (Cruet-Burgos et al., 2020, Cardoso et al., 2015, Fernandez et al., 2008). For comparison, maize lines typically have <2 μg/g of provitamin A carotenoids, and biofortification efforts have succeeded in increasing lines to 15 μg/g or more of provitamin A carotenoids (Prasanna et al., 2020). Studies have confirmed that there is quantitative variation in the sorghum grain carotenoid levels between different varieties, which is necessary for breeding higher concentrations. In the absence of variation, biotechnological strategies must be used. However, traditional breeding is a slow process, taking up to ten to twenty years to develop elite lines that farmers will use. Genomics-enabled breeding strategies have the potential to accelerate the traditional breeding process to produce elite biofortified sorghum lines within a few years (Diepenbrock & Gore, 2015). Quantitative trait loci (QTL) and genome-wide association studies (GWAS) have identified several loci underlying sorghum carotenoid variation (Cruet-Burgos et al., 2020, Fernandez et al., 2008). Genetic markers that can be used in breeding programs have been developed and are being tested in breeding programs in Haiti and Senegal (D. Rhodes, personal communication).

Since there can be tens to hundreds of genes within a genomic region identified in a GWAS that could be linked to a single nucleotide polymorphism (SNPs), the causative gene cannot be definitely identified, so candidate genes must be further analyzed to determine their function in the pathway (Cruet-Burgos et al., 2020). Of the SNPs identified by Cruet-Burgos et al, several were located near candidate genes that were also identified in GWAS of maize (*Zea mays* L.) and Arabidopsis (*Arabidopsis thaliana*). The most notable was an SNP within the sorghum zeaxanthin epoxidase (ZEP) gene, which is orthologous to both maize and Arabidopsis (Gebremeskel et al., 2018, Gonzalez-Jorge, 2016). In the Arabidopsis study, ZEP mutants lacking these genes were developed and produced a 6-fold increase of carotenoids in the seeds. Through gene expression experiments, variation in ZEP expression in Arabidopsis was shown to affect the composition, stability, and total quantity of carotenoids. The authors hypothesized that ZEP is an upstream gene in the carotenoid pathway that controls which carotenoids are targeted for degradation by the CCDs (Gonzalez-Jorge et al., 2016). This study confirms that the ZEP gene is a causal gene underlying carotenoid variation in Arabidopsis and supports the hypothesis that it is likely the causal variant identified in the sorghum carotenoid GWAS.

To build a deeper understanding of the genetic architecture of sorghum carotenoids the transcriptomics of the MEP precursor, biosynthesis, and degradation genes were evaluated for differential expression between high and low carotenoid lines and for their correlation to carotenoid concentrations throughout grain development (Cruet-Burgos et al., 2022). Several carotenoid pathway genes were differentially expressed between high and low carotenoid lines throughout development. Interestingly, at grain maturity most carotenoid pathway genes were more highly expressed in the high carotenoid lines compared to the low carotenoid lines. Notably, however, ZEP was not differentially expressed between high and low carotenoid lines.

This was a surprising result since the authors had previously identified it as a strong candidate underlying sorghum carotenoid variation (Cruet-Burgos et al., 2020 and 2023), and expression differences had been found to underlie carotenoid variation in Arabidopsis (Gonzalez-Jorge et al., 2016) and maize (Vallabhaneni and Wurtzel, 2009). However, the authors did identify several carotenoid pathway genes that were differentially expressed throughout grain development and that correlated with carotenoid concentrations, and these could be further explored as potential candidates to be used in marker-assisted selection.

A potential next step for studying the candidate genes identified by Cruet-Burgos (Cruet-Burgos et al., 2023) is to evaluate their expression across tissues. If individual genes have common genetic regulation across tissues, then it is possible that altering their expression in the grain might negatively affect their function in another tissue (antagonistic pleiotropy). Carotenoids have different functions in different plant tissues and are stored in tissue-specific plastids, so it can be hypothesized that their regulation is tissue-specific. In leaf tissue, carotenoids are produced in high concentrations and function in light-harvesting and photoprotection and are bound to chlorophyll-binding proteins in chloroplasts. In root tissue, carotenoids act as antioxidants and hormone precursors but are usually produced in low concentrations, with the exception of some starchy roots, and are often bound to lipid structures. In grain tissue, carotenoids are usually produced in small concentrations, act as antioxidants and hormone precursors, and are stored in amyloplasts, where starch storage takes place (Vishnevetsky et al., 1999, Yuan et al., 2015, Sun et al., 2018).

Additionally, little research has been conducted on the stability of stored carotenoids in sorghum grain. The sources of nutrient degradation before and after harvest must be identified and considered during biofortification efforts. Carotenoids in cereals can degrade due to a variety

of factors, including exposure to light, oxygen, and heat that increase lipid oxidation during storage and cooking (De Moura et al. 2015). Target countries have started to implement new storage containment that prevents oxygen exchange, light penetration, and pest infestations, but most storage facilities and transportation vehicles lack technology to control environmental conditions. Another potential source of degradation in the grain is after storage when biofortified products are processed and cooked.

The genetic controls underlying carotenoid degradation and the extent of degradation during storage and cooking are not well-studied in sorghum. When setting a target nutrient value for a biofortification breeding program, the potential degradation of the nutrient must be considered (Giuliano, 2017). To breed for favorable degradation alleles without antagonistic pleiotropic effects in sorghum, their gene expression, and the expression of the genes they interact with, must be compared across tissues. Additionally, to accurately establish a target nutrient value that accounts for degradation, carotenoid losses throughout various storage and cooking methods need to be evaluated.

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Chapter 2. Tissue-Specific Carotenoid Gene Expression: A Targeted Approach for Sorghum Grain Biofortification

Diets lacking carotenoid-rich foods can result in vitamin A deficiency (VAD), which is one of the most important public health concerns worldwide, particularly in sub-Saharan Africa (World Health Organization, 2009, Zhao et al., 2022). Carotenoids are a diverse group of compounds synthesized in all photosynthetic organisms and are also important dietary phytochemicals for humans (Meléndez-Martínez et al., 2022, Kean et al., 2007). Studies have shown that the high antioxidant activity of some carotenoids can reduce the risk of chronic and degenerative diseases (Sesso et al., 2004, Mozaffarieh et al., 2003), whereas the provitamin A activity of other carotenoids can reduce the risk of VAD. High concentrations of the provitamin A carotenoids (pVAC) β-carotene, α-carotene, and β-cryptoxanthin are found in many fruits and vegetables, but fruits and vegetables are inaccessible to many global populations or only available during certain times of the year. Staple cereal grains, therefore, provide a majority of the vitamin A consumed by humans. However, most cereals have low concentrations of provitamin A carotenoids in their edible portions so large quantities of these foods must be consumed to meet dietary needs (Rao, & Rao, 2007).

Sorghum (*Sorghum bicolor*) is a staple cereal crop that provides the majority of total caloric intake in many regions with high incidence of vitamin A deficiency, as only trace amounts of pVAC are present in the grain (Cruet-Burgos et al., 2020). Global high-dose vitamin A supplementation programs have been the primary intervention to address VAD, but these programs do not always reach some of the most vulnerable population in rural areas, and due to the COVID-19 pandemic, only 11 of the 64 target countries were able to fully supplement 80%

of the deficient population in 2020 (UNICEF, 2021). In the wake of climate change, crops such as maize are predicted to decrease in yield, making sorghum's ability to produce grain in hot, drought, and saline conditions critically important for providing food security (Ciampitti & Prasad, 2019). Increasing the sorghum grain pVAC content through biofortification breeding can provide additional supplementation to a broader population. However, the regulation of carotenoids throughout the plant must be understood in order to effectively breed for them in the grain, without negatively affecting their function in another tissue (antagonistic pleiotropy).

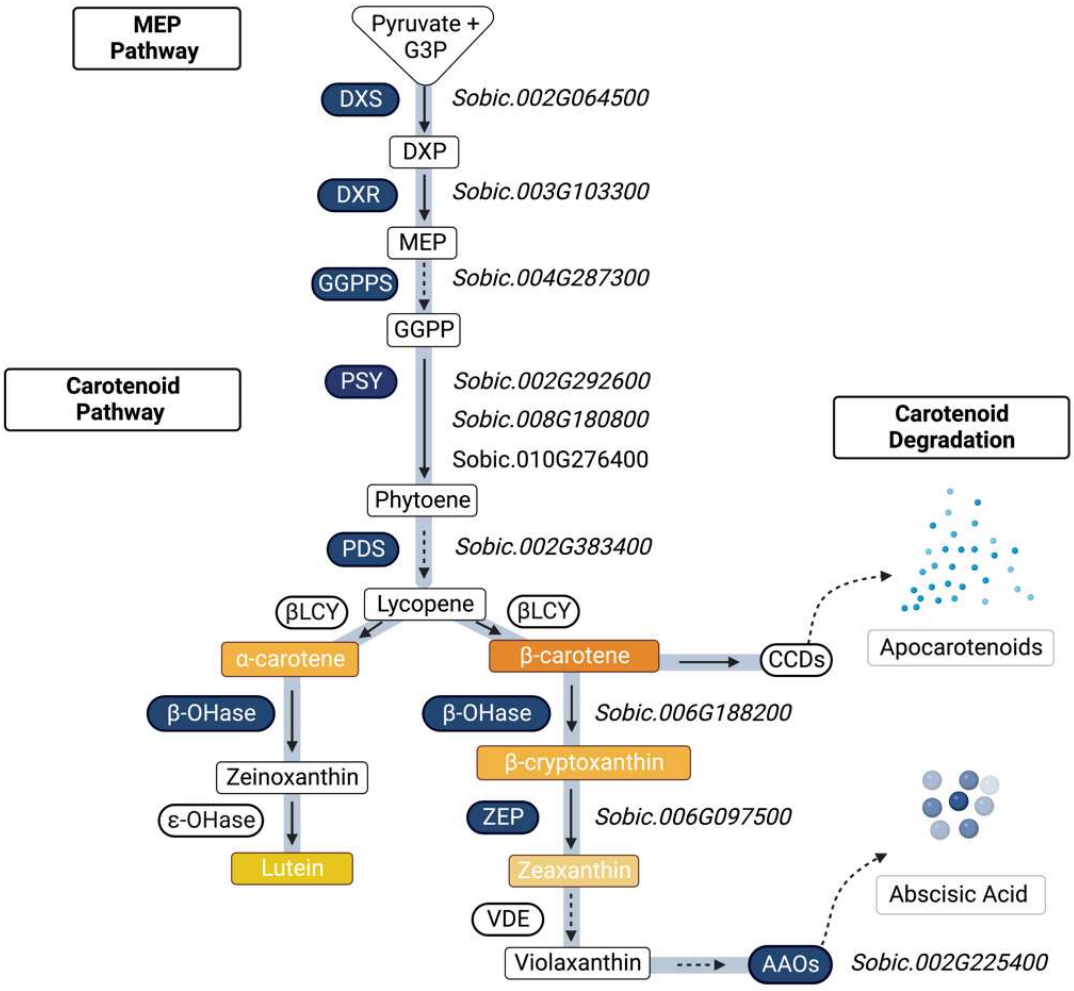

Figure 2.1 Genes of interest in the MEP and Carotenoid Pathways. Biosynthesis and degradation occur within plastids of plant cells, and degradation products (apocarotenoids) are sequestered to other plant tissues. Carotenoids of interest are colored in shades of yellow and orange rectangles, and carotenoid genes of interest are colored in blue ovals.

Final carotenoid concentrations in the grain are a result of interactions between the carotenoid precursor, biosynthesis, and degradation pathways (Fig. 2.1). The methylerythritol phosphate (MEP) precursor pathway is localized in plastids and synthesizes precursors used in several terpenoid biosynthesis pathways, including chlorophylls, tocochromanols, and carotenoids. Competition for MEP-derived precursors represents a bottleneck in carotenoid biosynthesis. The MEP pathway begins with the condensation of glyceraldehyde-3-phosphate and pyruvate, and through a series of enzymatic reactions produces two C5 isoprenoids, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). IPP and DMAPP then condense to finally produce the C20 compound geranylgeranyl diphosphate (GGPP), which can then be converted into phytoene-by-phytoene synthase (PSY), in the first committed step of the carotenoid pathway. Increased PSY expression is known to increase all downstream carotenoid concentrations, so PSY is often targeted for biofortification. Phytoene is eventually converted to lycopene production, at which point the pathway splits into the lutein branch and the zeaxanthin branch. Lycopene β-cyclase (βLCY) alleles that divert more substrate down the zeaxanthin branch can increase provitamin A concentrations, so βLCY is another target for biofortification efforts in some crops. β-carotene hydroxylase catalyzes the conversion of β-carotene to zeaxanthin. Finally, zeaxanthin epoxidase catalyzes the conversion of zeaxanthin, which is the first step toward abscisic acid biosynthesis.

The carotenoid pathway is a crucial metabolic pathway that is essential for plant development and survival. The pathway is active in various tissues throughout the plant, including the grain, leaves, and roots (Cazzonelli & Pogson, 2010). It is involved in a range of processes, including photosynthesis, photoprotection, and the production of hormones and other signaling molecules (Li, Vallabhaneni, & Wurtzel, 2008). The carotenoids themselves play

important roles in these processes, as well as serving as precursors for the production of other compounds such as ABA (Gallagher et al., 2004). Carotenoid-derived signaling hormones are essential to regulating both abiotic and biotic stressors throughout plant development, ABA is synthesized through the conversion of zeaxanthin to violaxanthin by ZEP, then a series of 9-cis-epoxy carotenoid dioxygenase (NCEDs) and abscisic aldehyde oxidases (AAOs) further catalyze reactions, resulting in the production of ABA (Ma et al., 2019). In cereal crops, evidence supports a metabolic feedback interaction through the expression of multiple tissue-specific genes for PSY that regulate the supply of MEP precursor products influencing the accumulation of both carotenoids and downstream apocarotenoids (compounds derived from carotenoids through oxidative cleavage) such as ABA (Cazzonelli & Pogson, 2010, Gallagher et al., 2004, Li, Vallabhaneni, & Wurtzel, 2008). Within this metabolic feedback regulation, the accumulation of pVAC β-carotene and β-cryptoxanthin could be targeted by finding allelic variants that increase or decrease the expression of enzymes throughout the pathway. A target of particular interest is the ZEP gene, which can be considered a provitamin A degradation enzyme. Therefore, understanding the carotenoid pathway and its regulation is critical for optimizing plant growth and health, as well as for developing new biofortified varieties.

Significant associations between genetic markers and carotenoid concentrations have been identified by GWAS in the precursor MEP pathway and carotenoid biosynthesis and degradation pathways (Cruet-Burgos & Rhodes, 2022). ZEP, which catalyzes the conversion of zeaxanthin to violaxanthin, appears to be a major gene-controlling variation in sorghum grain carotenoid compounds (Cruet-Burgos & Rhodes, 2022). Alleles that slow the rate of carotenoid degradation are another potential target for biofortification breeding. Transcriptomics analysis identified several candidate genes in the MEP precursor pathway, the biosynthesis pathway, as

well as the degradation pathways that were differentially expressed between high and low carotenoid accessions (Cruet-Burgos & Rhodes, 2022). Although genes underlying carotenoid variation in sorghum grain have been studied, an understanding of the expression of candidate genes in other sorghum tissues is limited. In tomatoes, differential expression of carotenoid candidate genes was identified in eight different plant tissues, and the authors concluded that transcriptional controls are an interconnected network of regulatory mechanisms (Koul et al., 2016). Evaluating sorghum carotenoid candidate genes identified as potential breeding markers and their expression in all plant tissues will show how modifying them could positively or negatively impact essential physiological functions.

To determine if breeding for high carotenoid alleles in sorghum grain without causing antagonistic pleiotropic effects is possible, this study evaluated the gene expression of candidate genes in the MEP pathway, and carotenoid biosynthesis and degradation pathways both within and between root, leaf, and grain tissues, and between high and low carotenoid groups. Additionally, the concentration of pVAC β-carotene, as well as lutein and zeaxanthin, were quantified to compare concentrations within and between tissues, and between high and low carotenoid groups. We hypothesized that sorghum carotenoid pathway gene regulation is tissue-specific, so genes can be manipulated in the grain without altering function in other tissue. To evaluate this hypothesis, a study was performed to 1) quantify the expression of carotenoid candidate genes in grain, leaf, and root tissues; 2) compare gene expression of carotenoid candidate genes between and within tissues; and 3) compare gene expression of carotenoid candidate genes in tissues between high versus low carotenoid concentration genotypes. Understanding the tissue-specific regulation and subsequent accumulation of carotenoids within tissues will advance the breeding scheme for sorghum carotenoid biofortification.

Methods

Plant Material and Tissue Collection

Six sorghum accessions with high (>1 μg/g: PI585348, PI585347, PI484369) and low (< 1 μg/g: PI511015, PI511018, PI510951) β-carotene concentrations were selected based on previous HPLC quantification (Cruet-Burgos, 2022). Using a complete randomized design (CRD), accessions were grown in triplicate for each tissue from April 2022 to Sep 2022 at the Plant Growth Facilities greenhouses located at Colorado State University. Grain, leaf, and root tissue were collected at grain maturity, signified by the formation of a black layer at the base of the seed (Fig. 2.2). Whole panicles were harvested, dried in an oven for 24 hours, threshed, and then flash-frozen in liquid nitrogen. Whole flag leaves were collected and immediately flash-frozen in liquid nitrogen. Roots were cut from the plant and soil was washed away from the roots using water under a faucet for 1 minute. Washed roots were dried on paper towels, and then a ~6-inch section in the middle of the root crown was cut and immediately flash frozen, as described in Okamura et al. 2021. Samples were then stored at -80°C until further application.

Figure 2.2 Grain, leaf, and root tissue samples from A) high carotenoid accession PI585348 B) low carotenoid accession PI511015.

RNA Extraction and Purification

An SDS-LiCl method (Vennapusa et al., 2020) with some modifications was used to extract total RNA from sorghum grain, leaf, and root tissue samples. 100 mg of tissue, 600 µL extraction buffer (100mM Tris-HCL (pH=8), 25 mM EDTA 2Na, 2.5% PVP, 2.5 M NaCl, 2.5% β-Mercaptoethanol in DEPC-water), and 2 grinding beads (Daisy, Zinc-plated, 4.5mm) were added to a 2 mL tube and ground using the Bead Ruptor Elite (Omni International,

Kennesaw, GA) for 30 seconds at 4 m/s. Next, the 2 mL tubes were placed in ice, an additional 500 µL of extraction buffer was added to each tube and then tubes were vortexed for 5 min. SDS was then added and the cleaning steps of the extractions were followed according to Vennapusa et al. 2020. Resuspended RNA was then treated as instructed with TURBO DNA-free™ Kit (Thermo Fisher Scientific, Vilnius, Lithuania), and this step was used to normalize RNA concentrations across tissue samples of each genotype by using the sample with the lowest RNA concentration. The integrity of RNA was tested on a 1% agarose gel stained with ethidium bromide to evaluate the 28S and 18S rRNA bands. RNA purity was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Vilnius, Lithuania) to measure the asorbance ratios at 260/280 nm and 260/230 nm with >1.5 accepted as pure RNA, and a concentration >30 ng/µL was accepted.

Primer Design

Genes for this study were selected based on candidate gene results from genomic mapping and transcriptomics studies previously conducted by our research group (Cruet-Burgos 2022a, Cruet-Burgos 2022b). Sequences were obtained from Phytozome (http://www.phytozome.net) using the sorghum reference genome v3.1.1 and entered in the PrimerQuest Real-Time PCR Design tool (Integrated DNA Technologies, Coralville, Iowa) to create forward and reverse oligonucleotides. The parameters set for the primer design were set according to Thornton (Thornton & Basu, 2011), and included the primer length, melting and annealing temperatures, product size, GC concentration %, and repeats. Once primer options were designed, the NCBI BLAST® service was used to identify the sequence pairs that only coded for our intended gene target. Reference genes Eukaryotic Initiation Factor 4A-1(EIF4a), and Serine/threonine-Protein Phosphatase (PP2A) were selected based on research in which

multiple reference genes were tested under various experimental conditions (Sudhakar et al., 2016). EIF4a and PP2A were found to be the most stably expressed across tissue types and environmental conditions. Primer sequences were ordered through Integrated DNA Technologies according to the selected primer pairs for our genes of interest and reference genes (Supplementary Table 1). Primer efficiency was evaluated by running a three-point 1:10 dilution curve and then calculating efficiency percentage through fold increase per cycle (Ruijter et al., 2021). Primer efficiencies were used to correct Ct values using the Design and Analysis Software v1.5.2 on the QuantStudio 3 (Thermo Fisher Scientific, Vilnius, Lithuania).

RT-qPCR

DNase-treated samples were converted to cDNA using qScript cDNA SuperMix (Quantabio, Beverly, Massachusetts). An interlacing dye assay using PerfeCTa® SYBR® Green FastMix® (Quantabio, Beverly, Massachusetts) was used on a 96-well plate, and reactions consisted of ten genes of interest, the two reference genes, and a no-template control, all run in technical triplicates. A cycling qPCR protocol was run on the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Vilnius, Lithuania), with a 95 °C denaturing step for 15 seconds followed by a 55 °C annealing step for 15 seconds. The denaturing and annealing steps were cycled 40 times and data were collected at the end of every annealing step. The fluorescence threshold was automatically set above the background noise, and raw cycle threshold (Ct) scores were recorded by QuantStudio once fluorescence exceeded the threshold. Relative gene expression was calculated using Ct scores for every gene of interest using the delta Ct method. The Ct value for each gene was subtracted from the mean Ct value of the reference genes EIF4a and PP2A in the same sample. The ΔCt values were then converted to relative

expression values using the $2^{-\Delta CT}$ formula. To normalize the relative expression values, the maximum relative expression across all samples and genes was determined, and each relative expression value was divided by the maximum value. The normalized relative expression values range from 0 to 1, with higher values indicating higher expression and lower values indicating lower expression.

Carotenoid Extractions

To avoid carotenoid degradation throughout the extraction process, samples were kept in dark conditions or under yellow light. All frozen tissue samples were lyophilized at -50 °C and 0.0 mbr using the FreeZone Freeze Dryer (Labconco, Kansas City, Missouri) for 72 hours, before returning to -80 °C. Lyophilized samples were extracted in randomized batches of 10 using a saponification method (Irakli et al., 2011) with modifications. In short, in a 2 mL tube, 400 µL of extraction buffer (absolute ethanol, 1 mg/ml BHT, and 0.03125 µg/mL apocarotenal) was added to 20 mg of ascorbic acid and ~20 mg of ground leaf sample, or ~100 mg of ground root and grain. To saponify the sample, 20 µL of potassium hydroxide solution (80% w/v in H2O) was added and vortexed, and then the tubes were placed in a water bath at 80 °C for 15 minutes, with a vortex every 5 minutes. Samples were centrifuged and the supernatant was collected. An additional 400 µL extraction buffer was added to the precipitate and vortexed. Samples were then centrifuged, and the supernatant was combined with the previously collected supernatant. Extracted samples were dried using nitrogen gas, and then resuspended in 100 µL of ethanol/ethyl acetate solution (50:50 v/v). The resuspended extracts were transferred to 11mm glass vial inserts (Thermo Scientific) encased within 2 mL glass snap amber HPLC vials (Thermo Scientific).

Carotenoid Quantification

A reverse-phase liquid chromatography method with modifications was used to quantify carotenoid concentrations (Dzakovich et al., 2022, Paine et al., 2005, Tan et al., 2017). For analysis, 6 µL of the extract was injected into a carotenoid C30 column (150 x 2 mm I.D. S-3 µm; YMC American, Inc.). The column temperature was maintained at 35 °C. Mobile phase A was methanol (95%): 1.0 M ammonium acetate (98:2 v/v), and mobile phase B was methyl tert-butyl ether, methanol, and 1.5% ammonium acetate (90:8:2, v/v/v). Carotenoids were resolved using a gradient: 100% A, 0% B; 3.5 min; 20% A, 80% B, 4.9 min; 0% A, 100% B; 1.7 min; 0% A, 100% B; 1.5min; 100% A, 0% B; 1.0 min; 100% A, 0% B; 6.0 min. Carotenoids were detected at 450 nm using the Flexar LC Quaternary Pump with a photodiode array detector (PerkinElmer, United States). Retention time and a six-point standard curve for β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin, and lutein were used to identify the peak area [µV∑s] of each carotenoid for all samples.

Analysis

The normalized expression of all carotenoid genes, and carotenoid concentrations values were grouped together for each accession and tissue. Data was aggregated by accession into high and low groups for concentrations, and genes were aggregated into precursor, biosynthesis, and degradation groups. Lutein, zeaxanthin, and β-carotene differences in concentrations and ratios between tissues and between high and low carotenoid genotypes were analyzed using two-way ANOVA and Tukey's post-hoc test in R. Correlations between carotenoid concentrations and gene expression levels were determined using Pearson's correlation coefficient in R. Differences in gene expression levels between tissues and between high and low carotenoid genotypes were

analyzed using two-way ANOVA in R. Gene correlations were also determined using Pearson's correlation coefficient in R. Significance was determined at $P < 0.05$.

Results

Carotenoid Concentrations in Sorghum Tissues

To test the hypothesis that sorghum carotenoid pathway gene regulation is tissue-specific, we predicted that carotenoid concentrations and ratios varied between tissues. Root, leaf, and grain tissues were analyzed in three accessions with high total carotenoids in the grain and three accessions with low total carotenoids in the grain. Lutein, zeaxanthin, and β-carotene were quantified within each tissue of the six accessions. The concentrations of lutein, zeaxanthin, and β-carotene varied between the different tissues and between high and low carotenoid groups (Supplemental Table 3). The average total carotenoid content was lowest in the root (1.73 μg/g), followed by grain (3.35μg/g), and then by leaf with the highest concentration (472.09 μg/g). In the root tissue, concentrations ranged from 0.45 - 0.55 μg/g of lutein, 0.51 - 0.55 μg/g of zeaxanthin, and 0.49 - 1.06 μg/g of β-carotene. In the grain tissue, the concentration of lutein ranged from 0.58 - 4.04 μg/g, zeaxanthin ranged from 0.35 - 2.26 μg/g, and β-carotene ranged from 0.29 - 1.31 μg/g. Lastly, in the leaf tissue, concentrations ranged from 261.29 - 373.25 μg/g for lutein, 5.73 - 10.86 μg/g for zeaxanthin, and 67.20 - 270.08 μg/g β-carotene. The tissue makeup of lutein, zeaxanthin, and β-carotene was markedly different between tissues (Fig. 2.3). In the root lutein, zeaxanthin, and β-carotene made up 29%, 28%, and 43% respectively of the total carotenoid concentration, whereas in the leaf lutein, zeaxanthin, and β-carotene made up 66%, 1%, and 33% respectively, and in the grain lutein, zeaxanthin, and β-carotene made up 48%, 32%, and 20% of the total carotenoid concentration respectively.

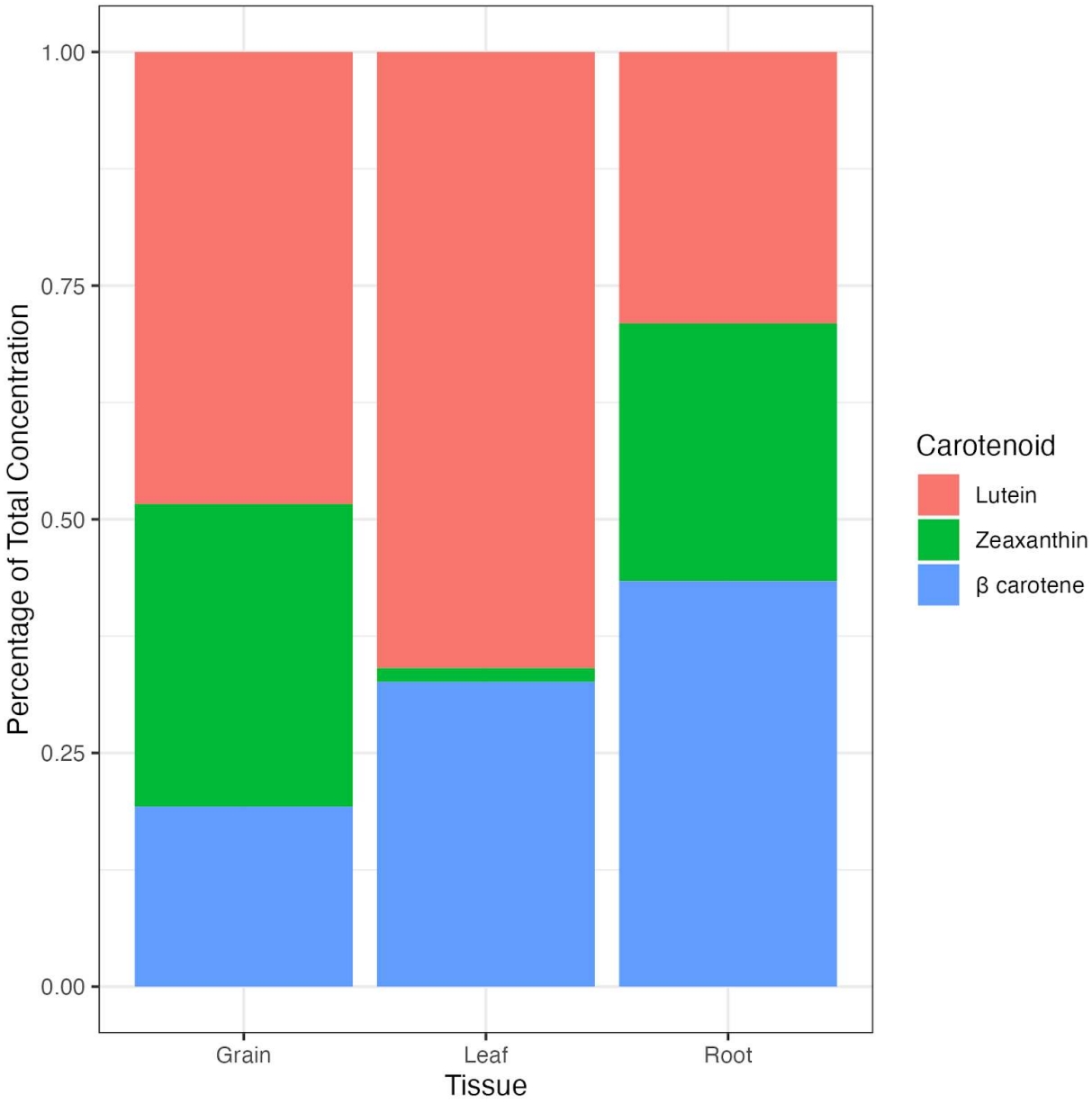

Figure 2.3 Carotenoid makeup within and between tissues. Percentages are of the average concentrations across all accessions.

Variations of carotenoid concentrations between high and low carotenoid genotypes between tissues can be seen in the large range of mean concentrations (Fig. 2.4). Genotypes were classified as high or low carotenoid genotypes based on the β-carotene content in the grain. In the grain, the high carotenoid group contained higher concentrations of each carotenoid

compared to the low concentration group. In the leaf, however, while lutein and zeaxanthin concentrations were higher in high carotenoid groups, β-carotene concentrations were lower in the high carotenoid group. In the root, there was little variation in lutein concentrations between high and low carotenoid groups, and zeaxanthin and β-carotene had higher concentrations in the high carotenoid group. These results show variations in carotenoid concentrations between tissues and between high and low carotenoid groups, although there was no statistical significance between the high and low groups of carotenoid concentration within the tissues, possibly due to a small sample size. Overall, the differences seen in concentrations across tissues for all genotypes provide support to the hypothesis that carotenoid regulation in sorghum is tissue specific.

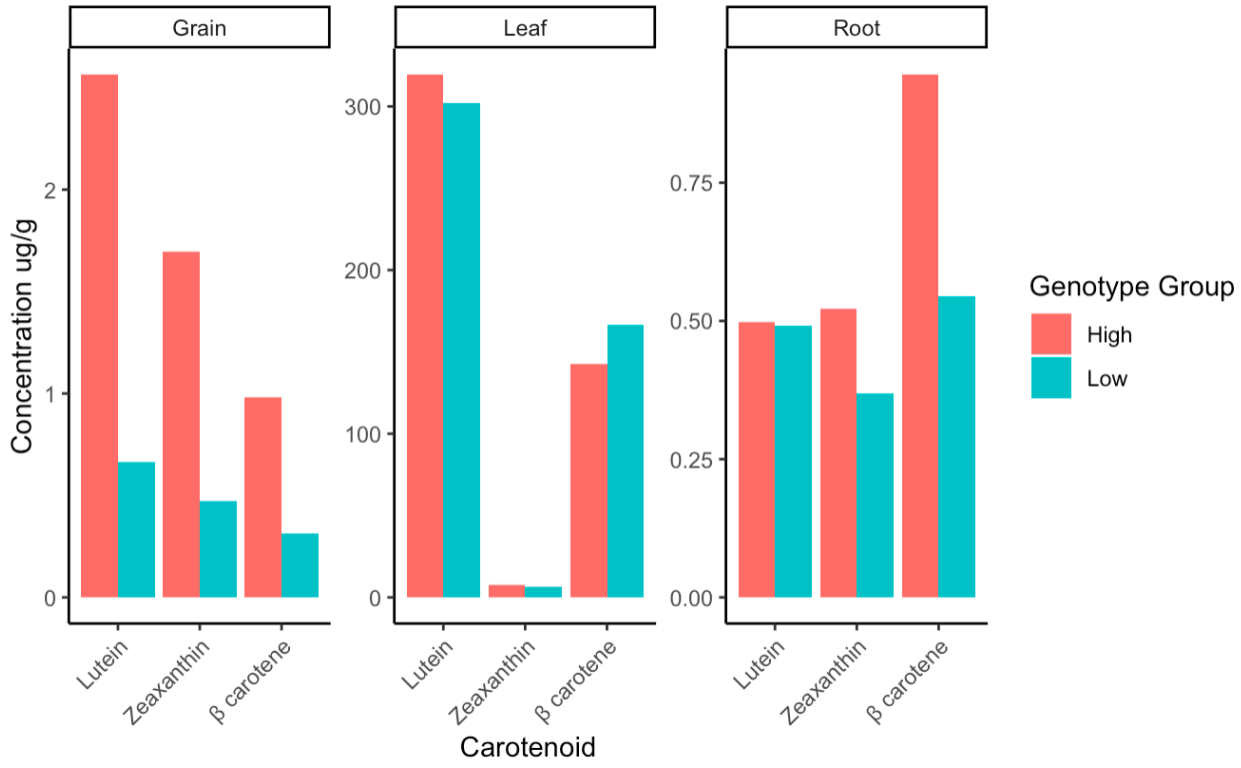

Figure 2.4 No evidence of pleiotropic effects across tissues. Bar graphs showing differences in total carotenoid concentrations (µg/g) between high ($n = 6$) and low ($n = 6$) accessions in grain, leaf, and root tissue. Note that scales are different between tissues.

Correlation of Carotenoid Concentrations in Sorghum Tissues

Next, we predicted that carotenoid concentrations were correlated within but not between tissues. Pearson's correlations were conducted between lutein, zeaxanthin, and β-carotene (Figure 2.5). Notably, in the grain tissue, there was a statistically significant, strong positive correlation between lutein and zeaxanthin ($r = 0.9$; $p < 0.000$), between lutein and β-carotene ($r = 0.94$; $p < 0.000$), and between zeaxanthin and β-carotene ($r = 0.94$; $p < 0.000$). In contrast, the leaf tissue showed no statistically significant correlations between carotenoids, except for an insignificant moderate correlation between β-carotene and lutein ($r = 0.38$; $p < 0.13$), and an insignificant moderate negative correlation between β-carotene and zeaxanthin ($r = -0.34$; $p < 0.18$). Due to the limited number of samples with detectable concentrations of lutein and zeaxanthin in the root tissue, no correlations could be determined within this tissue. Between tissues, there were no significant correlations, however, a moderate insignificant positive correlation was observed between grain and root β-carotene ($r = 0.58$; $p < 0.11$). Significant correlations within tissues, but not between tissues, suggests tissue-specific regulation of carotenoid genes.

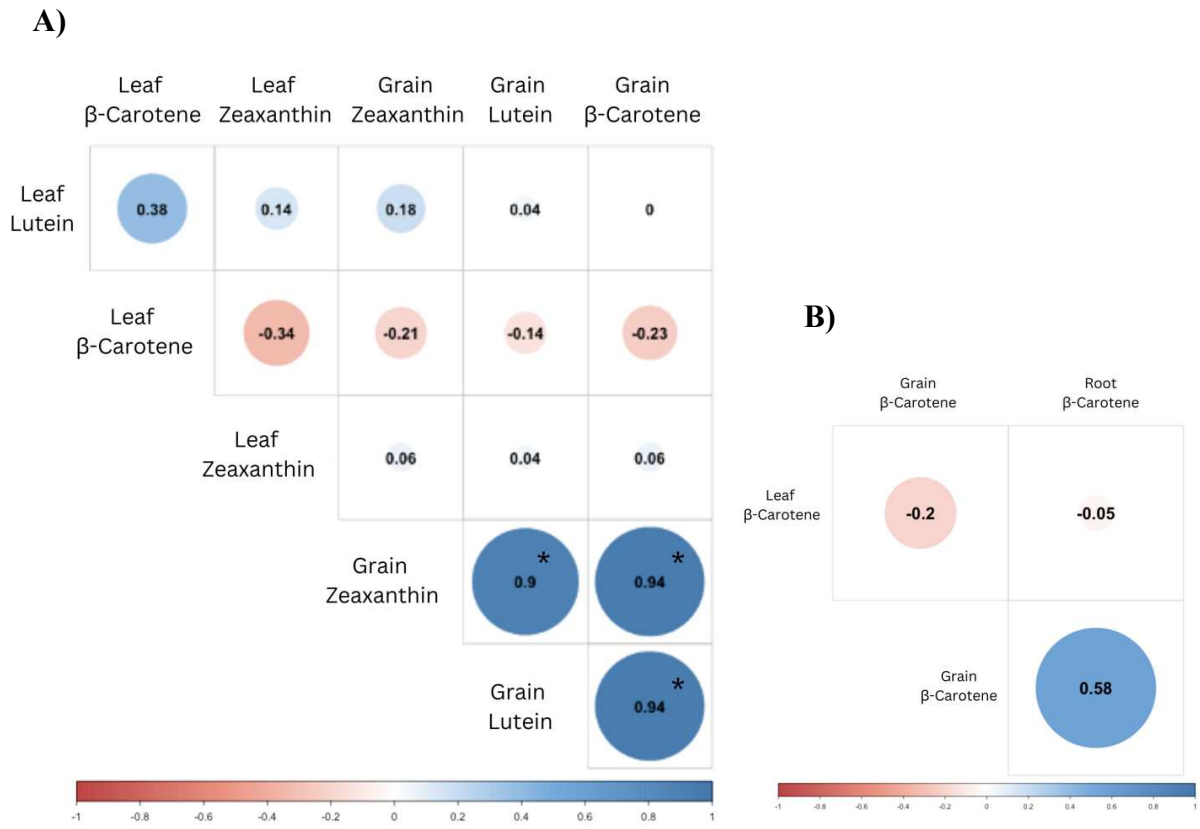

Figure 2.5 Correlation between tissues of A) lutein and zeaxanthin in grain and leaf, and B) β-carotene in grain, leaf, and root. Correlations range from -1.0-+1.0 with positive correlation indicated by the blue coloring, and negative correlation indicated by the red coloring. The intensity of the color is indicative of the correlation's significance with the * indicating a significant correlation ($p < 0.05$).

Carotenoid Gene Expression in Sorghum Tissues

To test the prediction that there is variation in carotenoid gene expression between tissues, we measured gene expression in root, leaf, and grain using RT-qPCR. We performed ANOVAs with tissue type and high/low carotenoid accession as factors. High and low groups did not show significant differences in mean expression levels for any gene, and tissue type was insignificant for most genes, except for PSY#3 and ZEP. Interaction between high/low groups and tissue was also not significant for any gene (Supplementary Table 2). Nonetheless, we observed differences in expression values across different genes between the tissues (Figure 2.6). When evaluating genes in groups of MEP precursor, biosynthesis, and degradation the grain

tissue had the highest expression in DXR, PDS, and BCH respective to each group. These genes were more highly expressed in high carotenoid grain than in low carotenoid grain (Figure 2.7). Overall, our results suggest that carotenoid gene expression is largely independent of tissue type and high/low carotenoid accessions. Still, some genes show tissue-specific expression and are more highly expressed in high carotenoid grain.

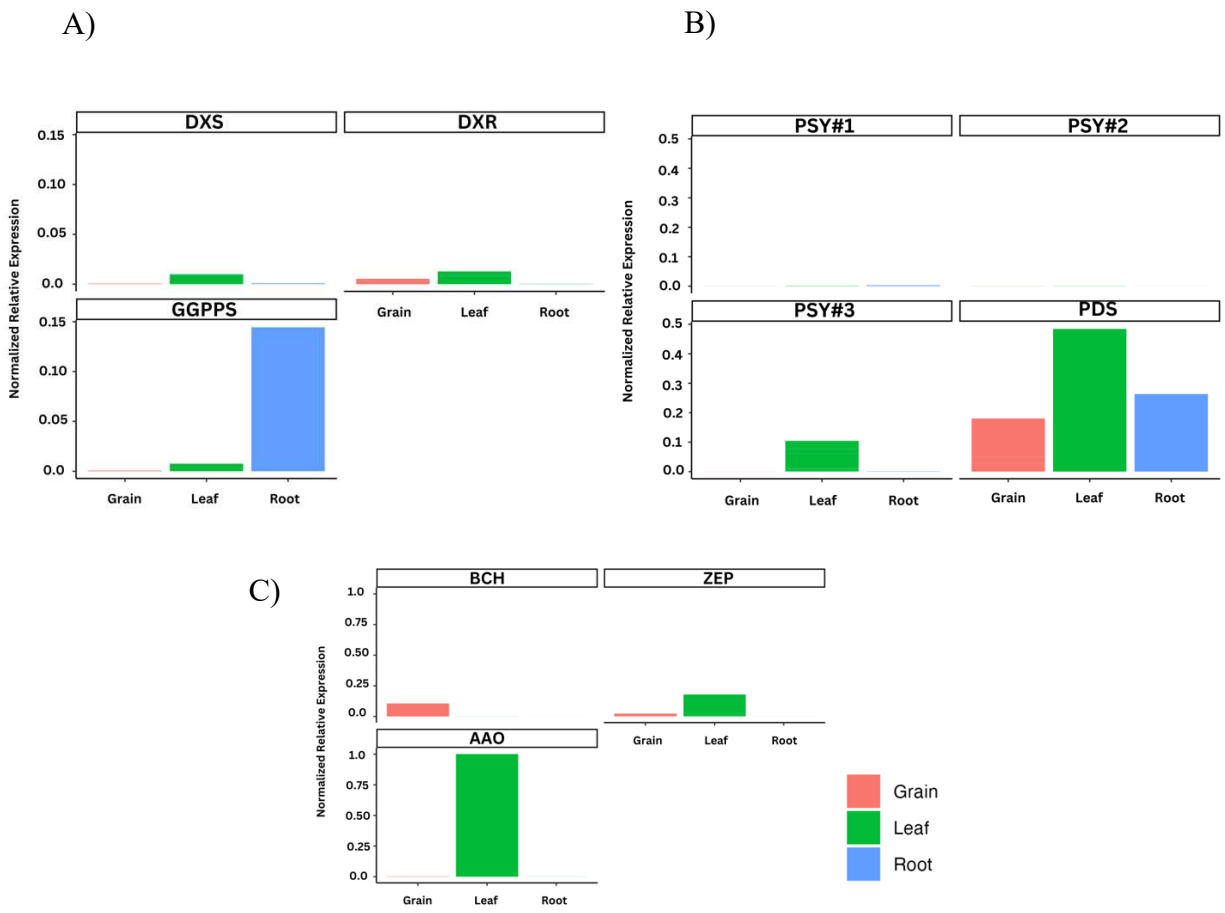

Figure 2.6 Normalized Relative Expression of A) MEP Precursor Genes, B) Carotenoid Biosynthesis Genes, C) Provitamin A Carotenoid Degradation Genes between grain, leaf, and root tissues.

A) B)

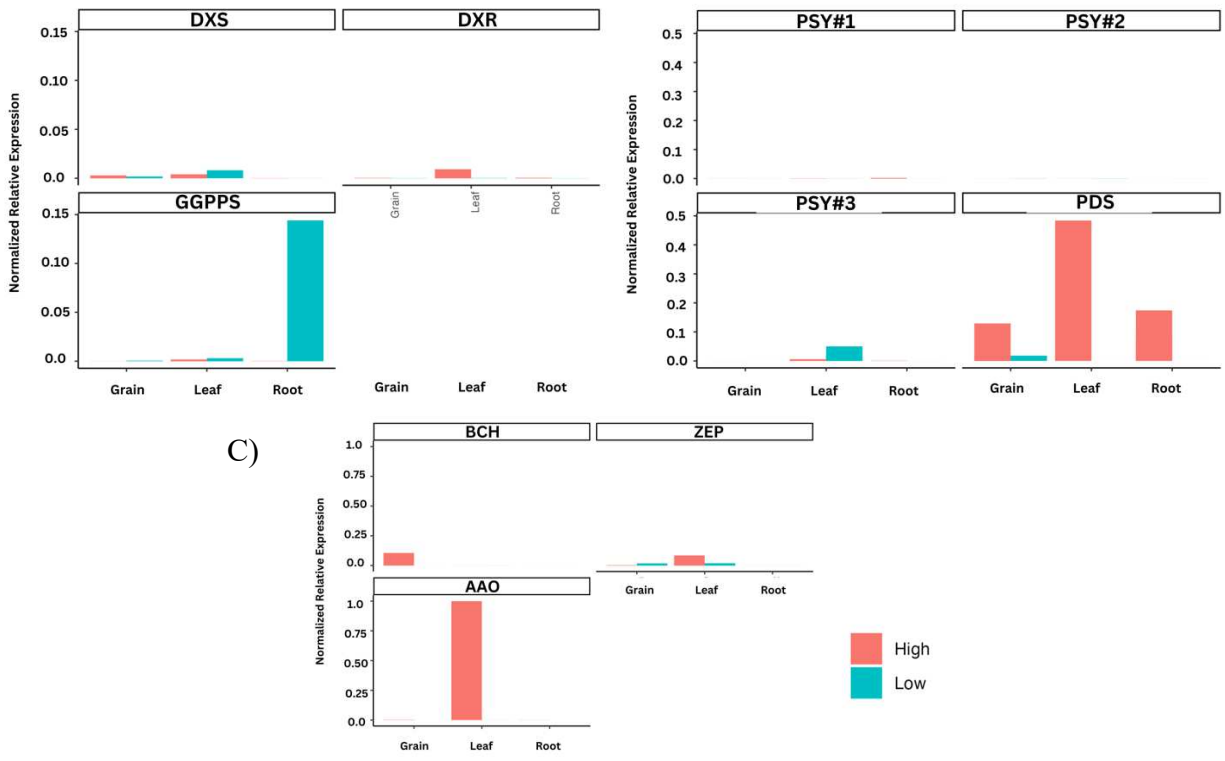

Figure 2.7 Normalized Relative Expression of A) MEP precursor genes, B) carotenoid biosynthesis genes, C) provitamin A carotenoid degradation genes between grain, leaf, and root tissues of high and low carotenoid concentration accessions.

Correlation of Carotenoid Concentrations in Sorghum Tissues

By comparing gene expression within and between tissues, we can identify genes with tissue-specific expression. Gene expression values for all genes for each tissue were used to evaluate the correlation within the grain, leaf, and root tissues as well as the correlations between each tissue pair. We first conducted Pearson's correlations between gene expression within tissues (Figure 2.8). Within the grain, there were significant correlations between DXS and DXR ($r = 0.83$; $p < 0.000$), and ZEP and GGPPS ($r = 0.99$; $p < 0.000$). Within the leaf, there were significant correlations between BCH and GGPPS ($r = 0.58$; $p < 0.02$), and DXS and PSY#1 ($r = 0.91$; $p < 0.000$). Root had the most gene correlations with significance between ZEP and DXS (r

= 0.73; $p < 0.004$), ZEP and PSY#1 ($r = 0.71$; $p < 0.004$), ZEP and PSY#2 ($r = 0.58$; $p < 0.03$), and PSY#1 and DXS ($r = 0.89$; $p < 0.000$).

Next, we conducted Pearson's correlations of gene expression between tissues (Figure 2.9). Between grain and leaf tissue, there was a significant correlation between the expression of seven gene pairs. BCH grain and PDS leaf ($r = 0.99$; $p < 0.000$), DXR grain and DXS leaf ($r = 0.83$; $p < 0.000$), DXS grain and DXS leaf ($r = 0.96$; $p < 0.000$), DXR grain and PSY#1 leaf ($r = 0.72$; $p < 0.001$), DXS grain and PSY#1 leaf ($r = 0.86$; $p < 0.000$), PSY#1 grain and DXS leaf ($r = 0.48$; $p < 0.04$), and PSY#1 grain and DXR leaf ($r = 0.64$; $p < 0.007$). Grain and root tissue had significant correlations between four gene pairs. BCH grain and ZEP root ($r = 0.54$; $p < 0.04$), PSY#1 grain and DXS root ($r = 0.74$; $p < 0.002$), PDS grain and PSY#3 root ($r = 0.66$; $p < 0.01$), and PSY#3 grain and BCH root ($r = 0.73$; $p < 0.004$). Lastly, leaf and root tissues had significant correlations between eight gene pairs. AAO leaf and DXR root ($r = 0.73$; $p < 0.01$), AAO leaf and PDS root ($r = 0.87$; $p < 0.004$), BCH leaf and GGPPS root ($r = 0.85$; $p < 0.000$), DXR leaf and PSY#2 root ($r = 0.91$; $p < 0.000$), DXR leaf and ZEP root ($r = 0.60$; $p < 0.03$), ZEP leaf and DXS root ($r = 0.80$; $p < 0.001$), ZEP leaf and PSY#1 root ($r = 0.89$; $p < 0.000$), and ZEP leaf and ZEP root ($r = 0.52$; $p < 0.07$). It should also be noted that many of these correlations within a tissue have genes within the same MEP precursor, biosynthesis, or degradation groups such as DXS and DXR being a part of the MEP precursor pathway. Alternatively, the correlation between tissues is more likely to involve two genes from different groups such as AAO from a degradation pathway and DXR from the precursor pathway.

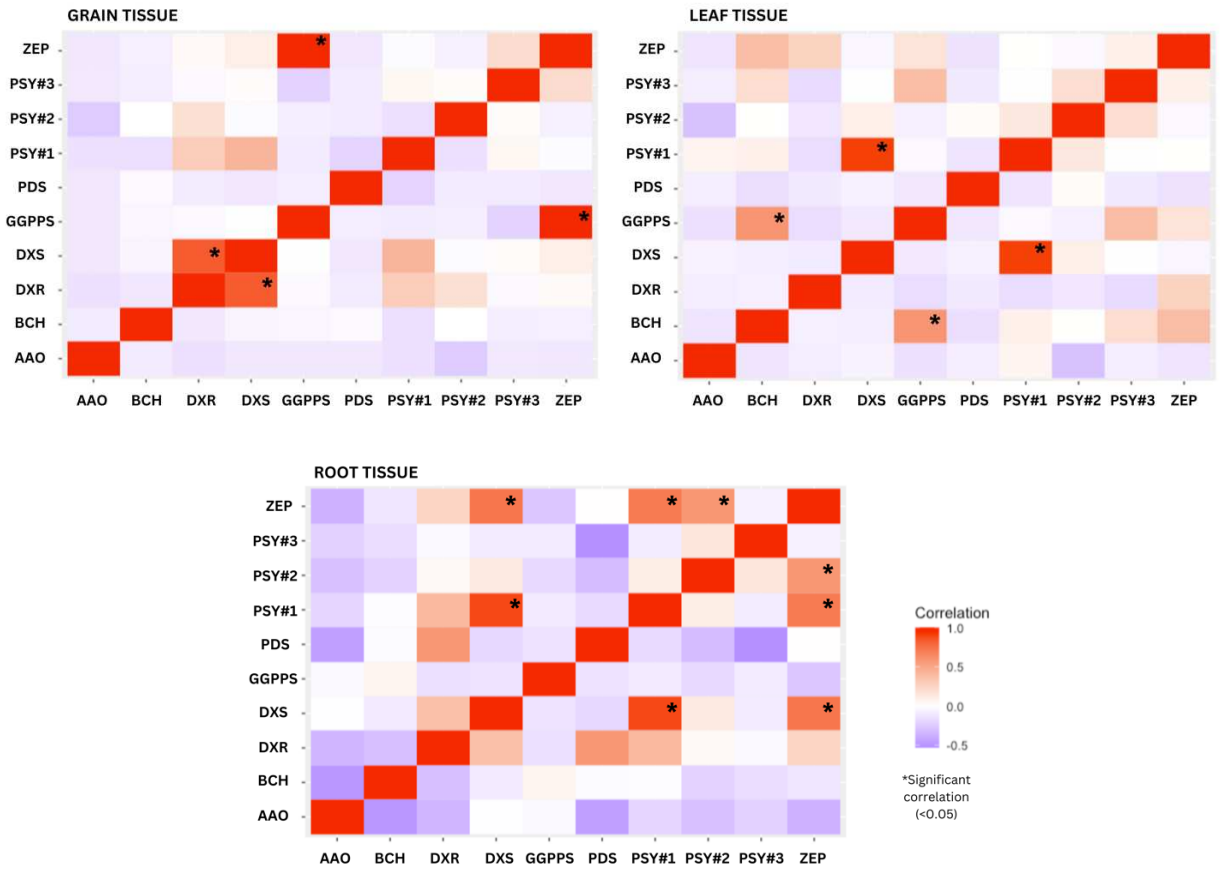

Figure 2.8 Correlation of carotenoid gene expression within tissues. Correlations range from -0.5-+1.0 with positive correlation indicated by the red coloring, and negative correlation indicated by the blue coloring. The intensity of the color is indicative of the correlation's significance with the * indicating a significant correlation ($p<0.05$).

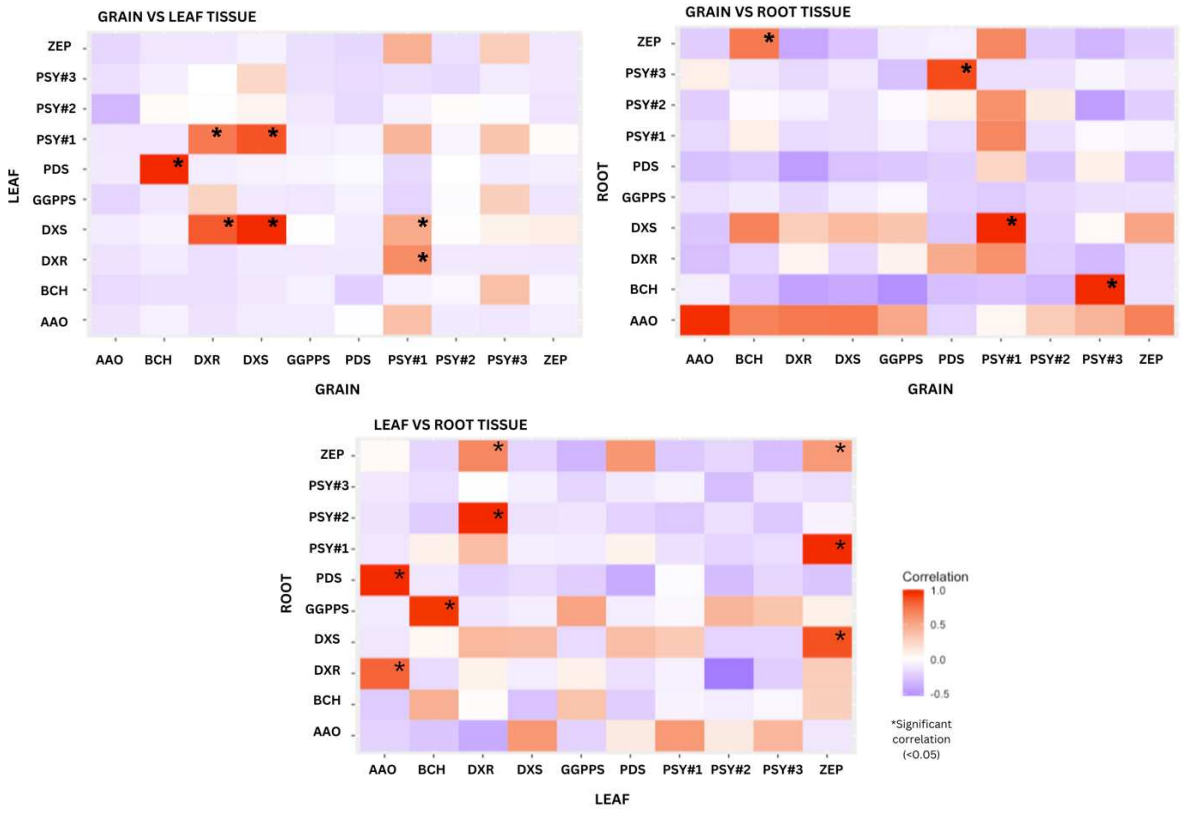

Figure 2.9 Correlation of carotenoid gene expression between tissues. Correlations range from -0.5-+1.0 with positive correlation indicated by the red coloring, and negative correlation indicated by the blue coloring. The intensity of the color is indicative of the correlation's significance with the * indicating a significant correlation ($p<0.05$).

Correlation Between Carotenoid Concentration and Gene Expression in Sorghum Tissues

Finally, we hypothesized that carotenoid pathway gene expression underlies carotenoid variation in each tissue. To test this hypothesis, we predicted that there would be significant correlations between carotenoid concentrations and gene expression values. For grain, there was an overall negative correlation between gene expression and carotenoid concentration for most of the genes and carotenoids, but none of the correlations were statistically significant (Fig. 2.9). In leaf tissue, BCH had high insignificant positive correlations with lutein and β-carotene, while AAO had a weak positive correlation with β-carotene. In contrast, PDS had strong insignificant negative correlations with lutein and β-carotene, but a significant positive correlation with

zeaxanthin. PSY#1 also had a moderate positive correlation with lutein and β-carotene. In the root there were significant negative correlations between DXR and lutein, and between DXR and β-carotene, while there was a significant positive correlation between AAO and lutein, and between AAO and β-carotene. In addition, there were significant negative correlations between PSY#1 and β-carotene, PDS, and lutein, and between PSY#3 and lutein. These results suggest that different carotenoids are regulated by different genes in a tissue-specific manner and that gene expression levels may be important in controlling carotenoid accumulation in each tissue.

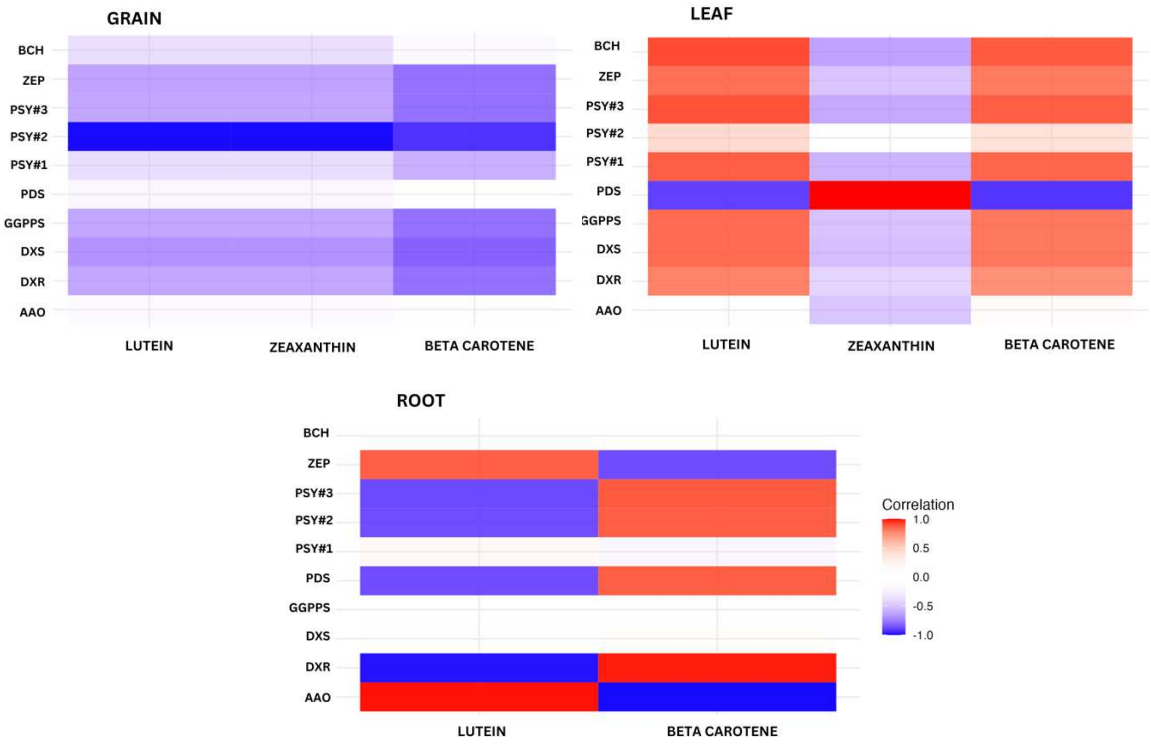

Figure 2.10 Correlation of carotenoid gene expression and carotenoid concentration within tissues. Correlations range from -1.0-+1.0 with positive correlation indicated by the red coloring, and negative correlation indicated by the blue coloring. The intensity of the color is indicative of the correlation's significance with the * indicating a significant correlation ($p<0.05$).

Discussion

Carotenoids are a group of pigments that are essential for plant function, and they also play important roles in human nutrition, particularly in providing provitamin A carotenoids. Sorghum is a staple crop in sub-Saharan Africa with VAD populations, and biofortification efforts aimed at increasing carotenoid content in sorghum have the potential to improve its pVAC content. In sorghum, there has been a recent focus on advancing provitamin A biofortification through molecular breeding, and genome-wide association studies (GWAS) of grain carotenoids in global germplasm (Cruet-Burgos et al., 2020) and transcriptomics analysis (Cruet-Burgos & Rhodes, 2022) have been used to identify genetic marker candidates. These studies have identified candidate genes and pathways involved in variation of carotenoid grain concentrations in sorghum, which can inform breeding efforts to develop biofortified sorghum varieties. The importance of understanding tissue-specific regulation of carotenoid concentrations in sorghum has the potential to inform the development of a targeted approach for biofortification. By identifying tissue-specific genes that regulate carotenoid biosynthesis and degradation, it may be possible to develop biofortified sorghum varieties with higher carotenoid content and improved nutritional quality. Additionally, investigating gene expression and carotenoid concentrations within tissues is crucial for identifying potential antagonistic pleiotropic effects.

Carotenoid Concentrations in Sorghum Tissues

Our results demonstrate that carotenoid concentrations vary between tissues, with β-carotene being the most abundant carotenoid in root tissue, while lutein and zeaxanthin were more abundant in leaf and grain tissue. Leaf tissue had the highest total concentration of

carotenoids, followed by grain, and then root. This is consistent with previous studies in other plant species that have also shown tissue-specific differences in carotenoid concentrations (Cazzonelli et al., 2010; Gonzalez-Jorge et al., 2013). The variation in carotenoid concentrations between tissues can be attributed to a variety of factors, including tissue-specific gene expression, metabolic activity, and transport mechanisms (Cazzonelli and Pogson, 2010). For example, the differences in carotenoid composition between grain and leaf tissues may be due to the differential expression of carotenoid biosynthesis genes in these tissues (Gonzalez-Jorge et al., 2013). Additionally, carotenoids may be metabolized differently in different tissues, which can also contribute to tissue-specific differences in carotenoid concentrations (Ruiz-Sola, & Rodriguez, 2012). Furthermore, environmental factors such as light intensity, temperature, and soil nutrient availability can also impact carotenoid concentrations in different plant tissues (Llorente et al., 2016; Coesele et al., 2008). In summary, the tissue-specific differences in carotenoid concentrations observed in our study may be due to a combination of genetic, metabolic, and environmental factors that impact carotenoid biosynthesis, concentration, and degradation in different plant tissues. Further research is needed to understand the specific mechanisms underlying the tissue-specific regulation of carotenoid concentrations in sorghum tissues.

Correlation of Carotenoid Concentrations in Sorghum Tissues

We observed tissue-specific correlations between carotenoids within tissues, with a strong positive correlation observed between lutein and zeaxanthin, and between lutein and β-carotene, and zeaxanthin and β-carotene in grain tissue. In contrast, no statistically significant correlations were observed between carotenoids in different tissues. The lack of correlations

between tissues suggests tissue-specific regulation of carotenoid concentration. These findings are consistent with previous studies that have suggested that tissue-specific regulation of carotenoid biosynthesis may be an important mechanism for controlling carotenoid concentration in different tissues (Cazzonelli & Pogson, 2010; Gonzalez-Jorge et al., 2013). The observed correlations between carotenoids within tissues suggest the existence of regulation of carotenoid biosynthesis within specific tissues. In grain tissue, the statistically significant strong positive correlations between lutein and zeaxanthin, and between lutein and β-carotene, and zeaxanthin and β-carotene may indicate a shared regulation of the biosynthesis pathways for these carotenoids in grain tissue. This is consistent with previous studies that have reported similar positive correlations between carotenoids in the same tissue (Muzhingi et al., 2008; Schaub et al., 2017). The moderate, insignificant positive correlation observed between grain and leaf β-carotene suggests that the regulation of β-carotene biosynthesis may be shared between these two tissues to some extent. However, the weak negative correlation between β-carotene and both zeaxanthin and lutein in leaf tissue may indicate that different regulatory mechanisms are in this tissue.

The insignificant correlations between β-carotene in leaf and root tissues suggest that the regulation of β-carotene biosynthesis may be distinct in these tissues. The absence of correlations between carotenoids in the root tissue is due to the fact that β-carotene is the primary carotenoid in the tissue and both lutein and zeaxanthin were at undetectable amounts in most samples. The high concentration of β-carotene in the percentage composition of root carotenoids is significantly higher than both leaf and grain β-carotene percentages and is evidence to further evaluate carotenoid concentration in root tissue. Overall, our findings suggest that carotenoid biosynthesis is regulated in a tissue-specific manner. The observed correlations between

carotenoids within tissues may reflect the shared regulation of biosynthesis pathways for these carotenoids, while the lack of correlations between carotenoids across tissues supports the notion of tissue-specific regulation of carotenoid biosynthesis.

Carotenoid Gene Expression in Sorghum Tissues

We found that carotenoid gene expression is largely independent of tissue type and high/low carotenoid accession. However, some genes showed tissue-specific expression, such as DXR, PDS, and BCH genes, which were more highly expressed in grain tissue and in high carotenoid grain than in low carotenoid grain. Tissue-specific expression of carotenoid biosynthetic genes is one of the mechanisms by which plants regulate carotenoid concentration. The expression of PSY, the first committed step in carotenoid biosynthesis, is highly regulated in a tissue-specific manner. In maize, PSY is expressed at higher levels in developing endosperm compared to other tissues, leading to the accumulation of carotenoids in the grain (Li, Vallabhaneni & Wurtzel, 2008). Similarly, in tomatoes, PSY is more highly expressed in the fruit tissue compared to other tissues, leading to carotenoid accumulation in the fruit (Enfissi et al., 2010).

Tissue-specific expression of other carotenoid biosynthetic genes, such as PDS, DXR, and BCH, has also been reported in various plant species. In Arabidopsis, PDS is more highly expressed in the chloroplast-rich tissues such as leaves and flowers, while BCH is more highly expressed in non-green tissues such as roots and flowers (Cazzonelli & Pogson, 2010). These findings suggest that tissue-specific regulation of carotenoid biosynthetic genes is an important mechanism for controlling carotenoid concentration in different tissues and developmental stages. Tissue-specific expression of carotenoid biosynthetic genes is a crucial mechanism by

which plants regulate carotenoid concentrations in different tissues and developmental stages. The identification of tissue-specific gene expression patterns and their regulation could provide insights into the molecular mechanisms controlling carotenoid concentration in sorghum and contribute to the development of varieties with enhanced carotenoid content.

Correlation Between Carotenoid Concentration and Gene Expression in Sorghum Tissues

Our results suggest that different carotenoids are regulated by different genes in a tissue-specific manner and that gene expression levels may be important in controlling carotenoid concentration in each tissue. This is consistent with previous studies that have shown that tissue-specific gene expression is important for regulating carotenoid concentration in different tissues (Ronen et al., 2000; Cazzonelli, & Pogson, 2010; Gonzalez-Jorge et al., 2013). Gene expression of PSY, ZDS, and ZEP were correlated with a carotenoid concentration in maize kernels, with PSY being the most important gene for carotenoid biosynthesis (Messias et al., 2014). Similarly, PSY1, LCYB, and BCH were the most important genes for carotenoid biosynthesis in tomato fruit, with different genes being important for carotenoid concentration in different tissues (Llorente et al., 2017). Our findings of tissue-specific correlations between carotenoids and gene expression further support the importance of tissue-specific regulation of carotenoid concentration in plants. The potential reasons for the correlations between and within the tissues may be due to the roles of the specific genes involved in carotenoid biosynthesis and their regulation in each tissue. The correlations observed within the grain tissue between DXS and DXR, and between ZEP and GGPPS may indicate the importance of the MEP pathway and GGPPS in carotenoid biosynthesis in this tissue. In contrast, the significant correlations between BCH and GGPPS, and between DXS and PSY#1 in the leaf tissue may suggest the importance of

these genes in carotenoid biosynthesis in leaves. Additionally, the significant correlations between ZEP and DXS, ZEP and PSY#1, and ZEP and PSY#2, and between PSY#1 and DXS in the root tissue may indicate the importance of these genes in regulating carotenoid concentration in roots.

The correlations observed between tissues may suggest the existence of regulation between different tissues. For instance, the significant correlation between BCH in the grain tissue and PDS in the leaf tissue may indicate that these genes are involved in regulating carotenoid concentration in both tissues. Similarly, the significant correlation between PSY#1 in the grain tissue and DXS in the root tissue may suggest a coordinated regulation of carotenoid biosynthesis between these tissues. Other studies have investigated the correlation between carotenoid concentration and gene expression in various plant species. A study in tomatoes found that high expression of PSY1 and PDS genes were associated with increased levels of lycopene and β-carotene, respectively (Li et al., 2011). These findings support our results that gene expression levels may be important in controlling carotenoid concentration in each tissue. In summary, the tissue-specific correlations observed between carotenoids and gene expression levels suggest the importance of tissue-specific regulation of carotenoid concentration in sorghum. Different carotenoids appear to be regulated by different genes in a tissue-specific manner, highlighting the complexity of carotenoid biosynthesis and regulation in plants. Further investigation of the functional roles of specific genes and their regulation in each tissue will enhance our understanding of carotenoid concentrations.

Conclusion

Our results support the hypothesis that most sorghum carotenoid pathway genes are regulated in a tissue-specific manner and can be manipulated in the grain without altering function in other tissues. We found that carotenoid concentrations vary between tissues, with β-carotene being the most abundant carotenoid in grain, while lutein and zeaxanthin were more abundant in leaf tissue. Our study also demonstrated tissue-specific correlations between carotenoids within tissues, suggesting tissue-specific regulation of carotenoid concentration. Furthermore, we identified tissue-specific gene correlations, indicating that certain genes are expressed specifically in certain tissues and that different carotenoids are regulated by different genes in a tissue-specific manner. These findings have important implications for the development of effective breeding strategies for carotenoid biofortification in sorghum. By identifying tissue-specific regulation of carotenoid concentration and the corresponding carotenoid biosynthesis genes, breeding programs can focus on enhancing carotenoid concentration in specific tissues while avoiding negative effects in other tissues. This could be achieved by selectively manipulating the expression of specific genes in the carotenoid biosynthesis pathway, such as DXR, PDS, and BCH, which were found to be more highly expressed in high carotenoid grain than in low carotenoid grain.

Our study is the first to investigate the tissue-specific regulation of carotenoid concentration in sorghum. However, our findings are consistent with previous studies that have shown tissue-specific differences in carotenoid concentration and gene expression in other plant species. These studies suggest that tissue-specific regulation of carotenoid biosynthesis may be an important mechanism for controlling carotenoid concentration in different tissues of various plant species. Overall, our study highlights the importance of understanding tissue-specific

regulation of carotenoid concentration and gene expression in sorghum. This knowledge can inform the development of effective breeding strategies for carotenoid biofortification, ultimately improving the vitamin A content of sorghum grain, thus giving deficient populations access to a nutritious diet.

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Chapter 3. Keeping Food Golden: The Role of Carotenoid Degradation in Sorghum Grain Biofortification

Sorghum is a staple cereal crop in many countries in Southeast Asia and Africa with high incidences of vitamin A deficiency (VAD). Pro-vitamin A carotenoids (pVAC) β-carotene, α-carotene, and β-cryptoxanthin are found in low concentrations within the sorghum grain, yet the dietary intake of plant carotenoids is essential to acquiring vitamin A in our diet as humans are not able to synthesize it and many people cannot consume enough preformed vitamin A in animal products to meet requirements (Rao & Rao, 2007). Biofortification breeding has the potential to increase carotenoid concentrations within the plant, however, it is important to assess carotenoids in the grain throughout harvesting, storage, processing, and cooking to establish the concentrations of pVAC at consumption (Cruet-Burgos & Rhodes, 2022). Abiotic factors such as temperature, humidity, and light conditions can degrade the quality of cereal grains post-harvest by altering nutritional composition (Pedreschi & Luire, 2015, Kumar & Kalita, 2017). Temperature is of specific interest in vitamin A biofortified sorghum since the target locations for biofortification have limited access to controlled storage environments. Inadequate post-harvest systems due to lack of environmental control, insufficient containment, and unsanitary facilities are the primary source of 20% of sorghum lost in African countries (FAO, 2022, Taleon et al., 2017), and it is unknown how these conditions affect carotenoid concentrations. Additionally, preparation of the sorghum grain is needed for consumption, and various processing and cooking methods could further alter the pVAC content. Understanding of the impact of each post-harvest step on the sorghum grain carotenoid content is limited but is

necessary in order to develop the tools needed to implement sorghum biofortification in VAD populations.

Carotenoid concentrations may be altered after maturity due to photo, thermal, and oxidative degradation, as well as, or in addition to exposure to acid, metals, and free radicals (Moura et al., 2015). Processing of sorghum grain post-harvest can potentially introduce one or more of these degradation effects, decreasing carotenoid content, and thus reducing the nutritional value. Traditionally, sorghum is harvested at maturity with a moisture content of around 25% and then dried to around 14% to inactivate degradative enzymes and reduce microbial activity (Sorghum Checkoff, 2023, Kramer 1977). Drying practices—either natural sunlight or artificial heat—is a possible source for initial decreases in carotenoid concentration. Once the ideal dry moisture content is achieved, the grain is threshed off the panicles and stored. Access to environmentally controlled storage facilities is often limited, and even storage containers to distribute sorghum grain are scarce. More commonly, large piles of sorghum grain are exposed to the environment where they sit in the heat and sunlight with no protection (Oirere, 2018). Designs for a storage bag are being developed to create a hermetic environment to reduce exposure to oxygen and to lower respiration, however, these bags will not regulate temperature and could allow light penetration, as they are not designed to prevent carotenoid degradation (FAO, 2022). A better understanding of the effects of post-harvest storage conditions on carotenoid degradation in sorghum grain will aid in developing new technologies that address current storage issues as well as provide protective measures for future biofortified products.

While storage presents many challenges in maintaining nutritional quality, the processing and cooking of sorghum grain also has the potential to alter carotenoid content. Carotenoids are distributed disproportionately throughout the grain due to the surrounding matrices that the

nutrients are embedded in. Milling could potentially increase the carotenoid content by breaking the matrix structure and thus making the carotenoids more bioavailable, however, the loss of cell integrity further exposes the carotenoids to oxidative stress (Trono, 2019). In VAD populations, milled sorghum flour is important for pasta and porridge consumed as staple cultural dishes, and whole grain is used as couscous (Fall et al., 2016). In a study comparing dry versus wet heat cooking methods in whole sorghum, the carotenoid concentration was essentially unaffected, with 99% retention in boiled grains, whereas dry heat cooking in the oven and frying pan decreased the concentration up to 85% (Cardoso, 2014). While most of the other water-soluble vitamins are lost during wet cooking methods, carotenoids are lipid soluble, reducing the loss in concentrations when cooked in water (Garg et al., 2021). The evaluation of unmilled sorghum grain and various cooking techniques has given a general insight into the possible alteration in sorghum grain carotenoid content, however, understanding the effects of milled flour in wet heat cooking methods will give better insights into the availability of carotenoids when using biofortified sorghum in dishes highly consumed in the target VAD populations.

To establish the points in post-harvest production of sorghum grain in which carotenoid concentrations could be altered, we measured carotenoid concentrations during pre- and post-harvest, storage, milling, and cooking. The concentration of pVACs along with lutein and zeaxanthin were quantified at each time point to evaluate any increases or decreases in content. We hypothesized that sorghum grain carotenoids decrease in content from harvest to the final cooked product, and that sorghum stored in cooler temperatures and boiled whole have less carotenoid degradation. To evaluate this hypothesis, a study was performed to 1) quantify the concentration of carotenoids after drying during harvest; 2) quantify and compare carotenoid

concentrations between 4℃ and 22℃ storage temperatures; 3) quantify and compare carotenoid concentrations between whole and milled sorghum in both storage groups; and 4) quantify and compare carotenoid concentrations between cooked whole grain, and traditional porridge made from milled grain. Evaluating the effects of traditional production practices on sorghum grain carotenoid content will advance the understanding of maintaining nutritional quality up until consumption and further establish biofortification as a possible solution to VAD.

Methods

Plant Material and Sample Collection

Three sorghum accessions (PI585348, PI585347, PI484369) with high β-carotene concentrations (>1 ug/g) were selected based on previous carotenoid quantifications (Cruet-Burgos, 2022). Using a complete randomized design (CRD), 9 plants for each accession were grown from April 2022 to Sep 2022 at the Plant Growth Facilities greenhouses located at Colorado State University. Panicles were harvested at grain maturity (formation of a black layer at the base of the seed) and dried in an oven for 24 hours at 48.8 °C. The 9 panicles per accession were divided into three groups of three panicles each, panicles were threshed, and the grain was pooled within each group. The three groups were considered three biological replicates for analysis. Samples were collected during pre-harvest at maturity (immediately before the panicle was cut), post-harvest (after the panicle was dried and before storage), post-storage (after 30 days at 4 °C or 22 °C storage temperatures), post-processing (after the grain was milled into flour), and post-cooking (after the whole grain was made into couscous and the milled grain was made into porridge). After the sample was collected at each time point it was flash-frozen in liquid nitrogen and stored at -80 °C.

PI#	Panicle	Grain
585369		
585348		

585347		

Figure 3.1 Grain samples from the accession 585369, 585348, and 585247

Storage and Cooking

Each triplicate group was split into two experimental groups, half being stored at 20 °C (room temperature) and the other half stored at 4 °C (refrigeration). Each group was stored at the respective temperatures for 30 days, then snap-frozen in liquid nitrogen and stored at -80 °C until cooking analysis was performed. The grain was slowly thawed at 4 °C for 1 hour before processing or cooking. Using the IKA 2900000 Economical Analytical Mill (Cole-Parmer,Vernan Hills, Illinois) grain was processed into fine flour. A modified method by (Kean et al., 2011) was used, and adjustments from the original method were made based on cook times and water volume needed to fully cook the grain. To cook the whole grain, 5 grams of grain and 80 mL of distilled water were added to a beaker on a hotplate set to 100 ℃. Once the water came to a boil, the beaker was covered in aluminum foil and the hotplate temperature was lowered to 85 ℃ to bring it to a simmer. The grain was fully cooked after approximately 60 minutes and

assessed by cutting into the grain to identify any raw portions. Porridge was prepared according to Lipkie et al., 2013 to reflect a traditional Tô dish made in West Africa. A slurry was made using 5 grams of milled grain and 10 ml of distilled water before being added to 10 ml of boiling distilled water on a hotplate and stirred for 3 minutes. After cooking, the thickened mixture was removed from the heat to cool to room temperature (22 ℃).

Carotenoid Extractions

To avoid carotenoid degradation throughout the extraction process, samples were kept in dark conditions or under yellow light. All frozen tissue samples were lyophilized at -50 ℃ and 0.0 mbr using the FreeZone Freeze Dryer (Labconco, Kansas City, Missouri) for 72 hours, before returning to -80 ℃. Lyophilized samples were extracted in randomized batches of 10 using a saponification method (Irakli et al., 2011) with modifications. In short, in a 2 mL tube, 400 uL of extraction buffer (absolute ethanol, 1 mg/mL BHT, and 0.03125 ug/mL apocarotenal) was added to 20 mg of ascorbic acid and ~50 mg of ground sample. To saponify the sample, 20 uL of potassium hydroxide solution (80 % w/v in H2O) was added and vortexed, and then the tubes were placed in a water bath at 80 ℃ for 15 minutes, with a vortex every 5 minutes. Samples were centrifuged and the supernatant was collected. An additional 400 uL extraction buffer was added to the precipitate and vortexed. Samples were then centrifuged, and the supernatant was combined with the previously collected supernatant. Extracted samples were dried using nitrogen gas and then resuspended in 100ul of ethanol/ethyl acetate solution (50:50 v/v). The resuspended extracts were transferred to 50 uL bottom spring glass vial inserts

(XPERTEK, St. Louis, Missouri) encased within 2 mL amber glass vials (XPERTEK, St. Louis, Missouri).

Carotenoid Quantification

A reverse-phase liquid chromatography method (Dzakovich et al., 2022, Paine et al., 2005, Tan et al., 2017) with modifications was used to quantify carotenoid concentrations. For analysis, 6 µL of the extract was injected into a carotenoid C30 column (150 x 2 mm I.D. S-3 µm; YMC American, Inc.). The column temperature was maintained at 35 ℃. Mobile phase A was methanol (95%): 1.0 M ammonium acetate (98:2 v/v), mobile phase B was methyl tert-butyl ether, methanol, and 1.5% ammonium acetate (90:8:2, v/v/v). Carotenoids were resolved using a gradient: 100% A, 0% B; 3.5 min; 20% A, 80% B, 4.9 min; 0% A, 100% B; 1.7 min; 0% A, 100% B; 1.5min; 100% A, 0% B; 1.0 min; 100% A, 0% B; 6.0 min. Carotenoids were detected at 450 nm using the Flexar LC Quaternary Pump with a photodiode array detector (PerkinElmer, United States), and quantified relative to six-point standard curves for β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin, and lutein.

Analysis

ANOVA was performed to determine the significance of the effects of post-harvest handling time points, storage temperature, and cooking style on carotenoid concentrations. Tukey's HSD test was used to compare the mean concentrations between different time points, storage temperatures, and cooking styles. The effects of genotype on carotenoid concentrations were also analyzed using ANOVA, and the interaction effect between genotype and other factors was tested. The statistical analysis was performed using R. Significance level was set at $p < 0.05$.

Results

Degradation in Post-Harvest Handling

To test the hypothesis that post-harvest handling of sorghum grain contributes to carotenoid degradation, we predicted that there would be a significant amount of degradation between the initial concentration at pre-harvest and each post-harvest handling step, as well as between each consecutive time point (Table 3.1). We conducted an ANOVA between all of the collection time points (preharvest, drying, storage, milling, and cooking). Significant degradation of each carotenoid was identified between preharvest and most timepoints, but not between each successive time point (Figure 3.1). The significance of post-harvest handling time points on carotenoid concentrations was also significantly affected by genotype, with PI585348 having higher carotenoid concentrations (Figure 3.2). The greatest reduction in concentration from pre-harvest to post-cook was observed for β-carotene, which decreased by 39%. Lutein and zeaxanthin also showed significant reductions in concentration after post-harvest handling, with decreases of 27% and 35% respectively, post-cook (Table 3.1).

The concentration of β-carotene in sorghum grain was significantly affected by both timepoint ($F = 7.368$, $p < 0.001$) and genotype ($F = 31.512$, $p < 0.001$), but there was no significant interaction effect between them. Tukey's HSD test revealed that pre-harvest had a significantly higher concentration of β-carotene compared to post drying, post mill, post storage, and post cook ($p < 0.05$). Between each successive time point, post drying had a significantly higher concentration compared to post mill ($p < 0.05$).

The concentration of lutein in sorghum grain was significantly affected by both timepoint ($F = 5.632$, $p = 0.002$) and genotype ($F = 62.762$, $p < 0.001$), but there was no significant interaction effect between them. Tukey's HSD test revealed that pre-harvest had a significantly

higher lutein concentration compared to post cook ($p = 0.01$), post mill ($p = 0.03$), and post storage ($p = 0.001$), but there were no significant differences between other timepoints.

The concentration of zeaxanthin in sorghum grain was significantly affected by both timepoint ($F = 7.120$, $p = 0.0004$) and genotype ($F = 53.044$, $p < 0.001$), but there was no significant interaction effect between them. Tukey's HSD test revealed that pre-harvest had a significantly higher zeaxanthin concentration compared to all other timepoints ($p < 0.01$), while there were no significant differences in zeaxanthin concentration between each successive timepoint.

Table 3.1 Concentration of carotenoids (β carotene, lutein, and zeaxanthin) at different time points (Pre-harvest, Post drying, Post mill, Post storage, and Post cook). The concentration is measured in µg/g, percent change of concentration forms pre-harvest, and significant differences between time points are indicated when $p < 0.05$.

Carotenoid	Timepoint	Concentration (µg/g)	Percent Reduction from Pre-harvest (%)	Significant differences ($p < 0.05$)
B-carotene	Pre-harvest	0.95	0	Pre-harvest > post drying, post mill, post storage, post cook
	Post drying	0.78	17	Post drying > post mill
	Post storage	0.66	31	-
	Post mill	0.50	47	-
	Post cook	0.58	39	-
Lutein	Pre-harvest	2.44	0	Pre-harvest > post cook, post mill, post storage
	Post drying	1.77	27	-
	Post storage	1.63	33	-
	Post mill	1.85	24	-
	Post cook	1.78	27	-
Zeaxanthin	Pre-harvest	1.68	0	Pre-harvest > post drying, post mill, post storage, post cook
	Post drying	1.19	29	-
	Post storage	1.17	30	-
	Post mill	1.22	27	-
	Post cook	1.10	35	-

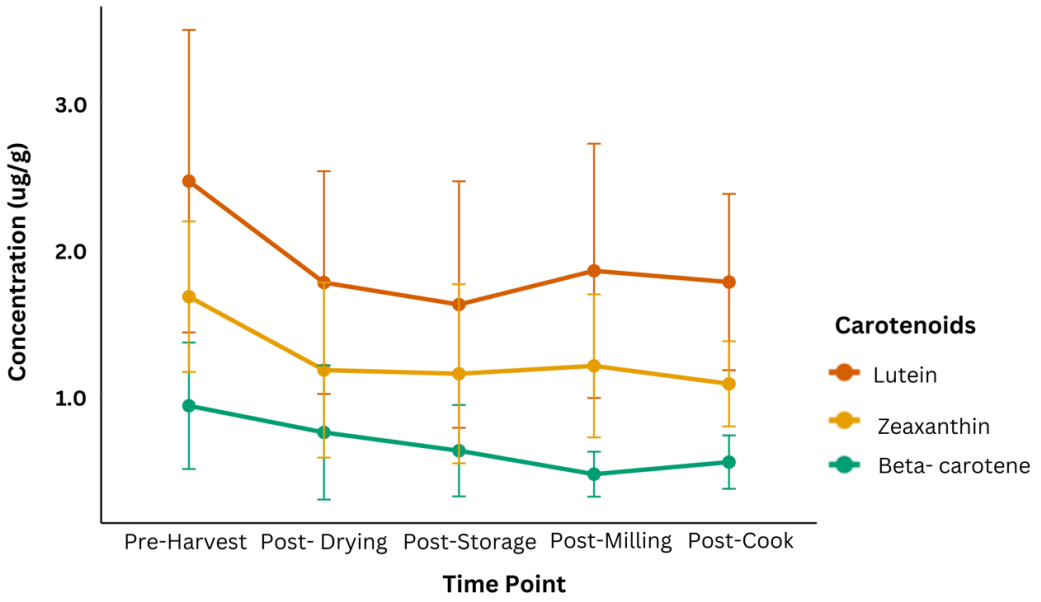
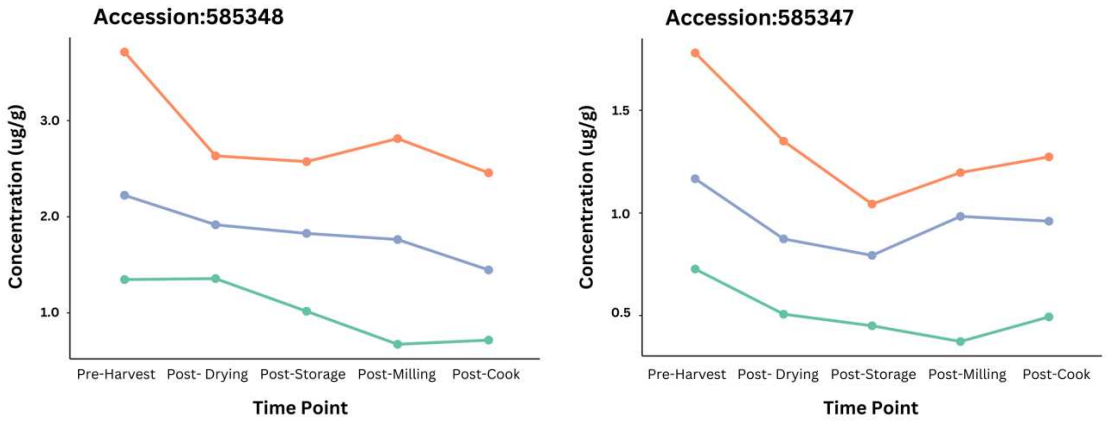


Figure 3.2 Changes in carotenoid concentration (mean and standard deviation) over five time points (Pre-Harvest, Post-Drying, Post-Storage, Post-Milling, and Post-Cook) for three carotenoids (Lutein, Zeaxanthin, and β -carotene).



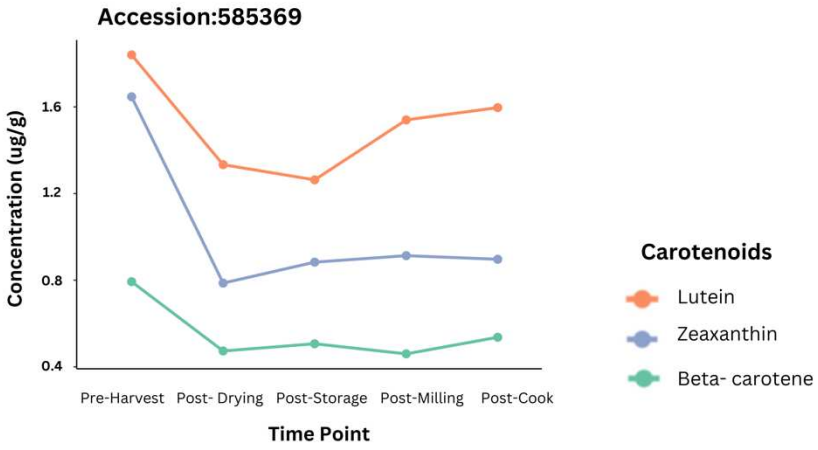


Figure 3.3 Changes in carotenoid concentration (mean and standard deviation) over five time points (Pre-Harvest, Post-Drying, Post-Storage, Post-Milling, and Post-Cook) for three carotenoids (Lutein, Zeaxanthin, and β -carotene) in three accessions (PI585348, PI585347, PI585369).

Degradation Under Varying Storage and Cooking Conditions

Next, we hypothesized that refrigerated storage and boiling whole grain slows degradation compared to room temperature storage and boiling milled grain. To determine if there was a significant difference in degradation between different storage conditions, carotenoid data were analyzed using an ANOVA. There were no significant differences in carotenoid degradation between 22°C and 4°C storage temperatures for all three carotenoids (Figure 3.3A). However, genotype had a significant effect ($p < 0.05$) on the concentration of all three carotenoids (Figure 3.3B). The interaction effect between storage temperature and genotype was not significant ($p > 0.05$).

The concentration of β-carotene in sorghum grain was significantly affected by genotype ($F = 3.898$, $p = 0.0496$), but not by the storage temperature ($F = 0.069$, $p = 0.80$) or the interaction effect between storage temperature and genotype ($F = 0.473$, $p = 0.63$). The concentration of lutein in sorghum grain was significantly affected by genotype ($F = 4.981$, $p = 0.03$), but not by the storage temperature ($F = 0.286$, $p = 0.60$). The interaction effect between storage temperature and genotype was not significant ($F = 1.676$, $p = 0.23$). The concentration of zeaxanthin in sorghum grain was significantly affected by

genotype (F = 4.770, p = 0.03), but not by the storage temperature (F = 1.249, p = 0.29). The interaction effect between storage temperature and genotype was not significant (F = 2.136, p = 0.16).

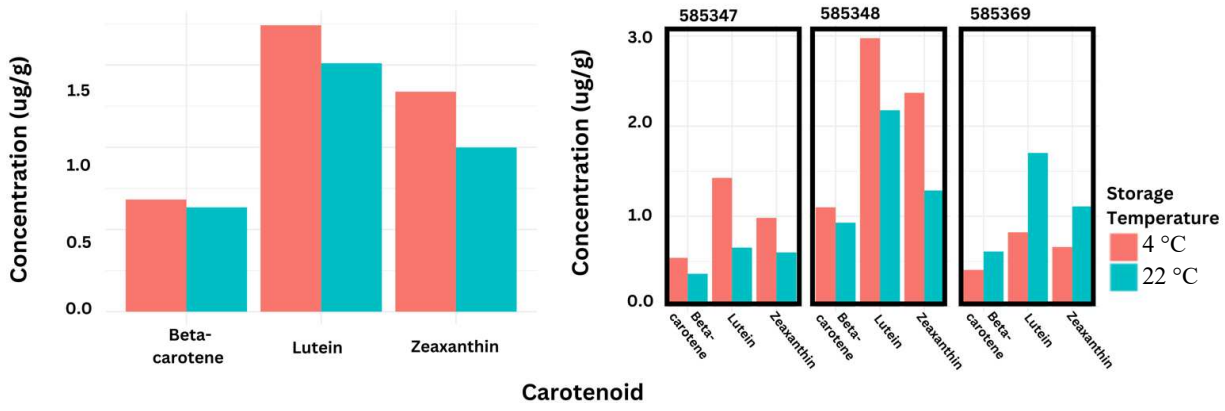


Figure 3.4 Comparison of average carotenoid concentration and individual genotype concentration between two storage temperatures (4C and 22C) for three carotenoids (Lutein, Zeaxanthin, and β-carotene).

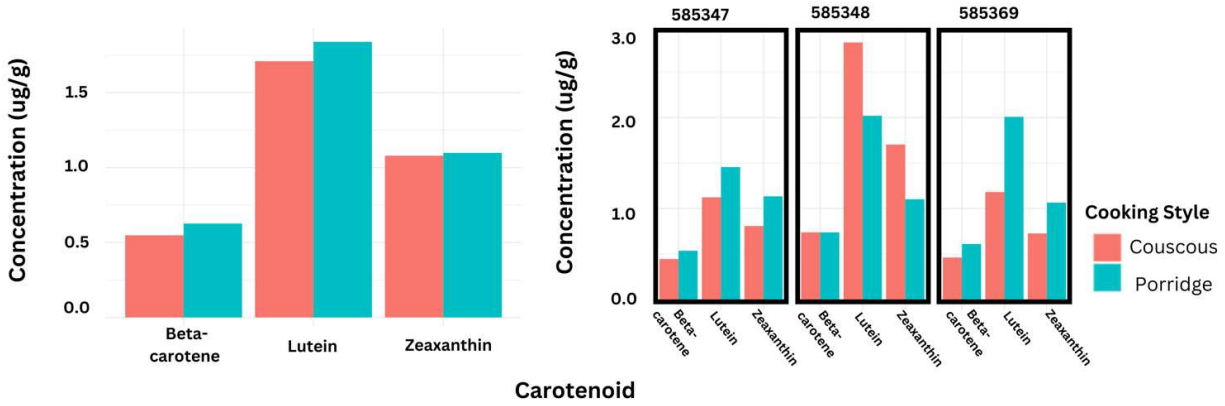


Figure 3.5 Comparison of average carotenoid concentration and individual genotype concentration between two cooking styles (Couscous and Porridge) after combining storage groups (4C and 22C) for three carotenoids (Lutein, Zeaxanthin, and β-carotene).

To determine if there was a significant difference in degradation between different cooking styles, the concentrations after cook for each storage group was averaged to analyze the two wet cooking methods. An ANOVA showed no significant difference in carotenoid degradation between couscous and porridge for all three carotenoids (Figure 3.4A). However, genotype had a significant effect ($p < 0.05$) on

the concentration of all three carotenoids (Figure 3.4B). The concentration of β-carotene in sorghum grain was significantly affected by genotype ($F = 5.175$, $p = 0.01$), but not by cooking style. The concentration of lutein in sorghum grain was significantly affected by genotype ($F = 4.154$, $p = 0.03$), but not by cooking style. The concentration of zeaxanthin in sorghum grain was significantly affected by genotype ($F = 14.204$, $p = 0.0001$) and the interaction effect between cooking style and genotype ($F = 11.508$, $p = 0.0004$), but not by the storage temperature.

There was significant degradation of carotenoids during post-harvest storage and processing, and there were variations in degradation depending on the genotype. The extent of degradation varies depending on the carotenoid and the specific post-harvest methods used. For example, zeaxanthin and β-carotene both experienced significant degradation during post-harvest storage at 4 °C and 22 °C, while lutein was less affected by storage. Additionally, all carotenoids experienced significant degradation during processing, with some methods, such as cooked couscous at 22 °C, resulting in greater degradation than others.

Discussion

Biofortification is a promising strategy for improving the nutritional quality of staple crops such as sorghum. Sorghum is an important food crop in many regions of the world, particularly in sub-Saharan Africa, where it is a key source of calories and nutrients. Biofortified sorghum with a high concentration of carotenoids can aid in a range of health benefits, including improved vision, reduced risk of chronic diseases, and enhanced immune function. However, there is still much to learn about how storage and processing conditions affect the concentration of carotenoids in biofortified sorghum grains, and how these grains can be optimally stored and cooked. The concentration of carotenoids in these grains can be affected by various factors, including genotype, storage temperature, and cooking style. Understanding the effects of post-harvest handling is crucial for developing strategies to maximize the nutritional value of biofortified sorghum and have accurate target concentrations. Previous studies have reported inconsistent findings on the effects of storage and cooking on carotenoid concentrations in sorghum

grains. Therefore, it is important to conduct further research to better understand the impact of these factors on carotenoid concentration in sorghum grain.

Post-Harvest Handling

The results of this study showed significant degradation of all three carotenoids (β-carotene, lutein, and zeaxanthin) during post-harvest handling. The greatest degradation was observed between pre-harvest and post-drying for β-carotene, while for lutein and zeaxanthin, the most significant degradation occurred between pre-harvest and post-storage. These results are consistent with previous studies that have shown significant degradation of carotenoids during post-harvest handling and storage in tomatoes, carrots, and peppers (Rath et al., 2020; Lisiewska et al., 2004; Schweiggert et al., 2007). The degradation of carotenoids during post-harvest handling is thought to be mainly due to exposure to light, heat, and oxygen, which can cause oxidation and other chemical reactions that degrade carotenoids (Rath et al., 2020), but further research in sorghum is needed to confirm the causes of degradation. Genotype also played a significant role in carotenoid concentrations, with some genotypes showing higher concentrations of carotenoids than others. The differences in carotenoid concentrations between genotypes may be due to genetic factors that regulate the degradation of the carotenoids (Vidhyavathi et al., 2014). The differences observed may also be attributed to the macronutrient matrix that encloses the micronutrients. The composition of the macronutrient matrix, including the type and number of proteins, fats, and carbohydrates, may vary between genotypes and influence the bioavailability and stability of micronutrients, such as carotenoids (Jintasataporn et al., 2012; Gemenet et al., 2016). A study found that variations in protein and fat content of different genotypes of sorghum resulted in differences in the bioavailability of carotenoids (Mashurabadet al. 2017). Similarly, there are reports of differences in the composition of macronutrients in maize grain that had a significant impact on the stability of carotenoids during storage (Ortiz et al.2016). Therefore, it is possible that the differences in carotenoid concentrations observed in our study may be due to differences in the macronutrient matrix of the different genotypes. However, further research is needed to fully understand the complex interactions between the

macronutrient matrix and the stability and bioaccessability of micronutrients in sorghum grain. Post-harvest handling and processing can significantly affect the concentration of β-carotene in sorghum grain. The concentration of β-carotene was found to decrease by 39% from pre-harvest to post-cooking, indicating that degradation during processing could significantly impact the target concentration of 4 μg/g set by the current sorghum biofortification program. A new β-carotene target concentration value of ~ 10 μg/g should be set to account for the degradation of carotenoids through post-harvest handling. A framework for setting target concentrations of biofortified crops based on the nutrient requirements of the target population, the dietary habits and practices, and the bioavailability and stability of the nutrient in the fortified crop can be used to establish a revised target concentration for β-carotene in sorghum grain (White and Broadley, 2009).

Storage and Cooking Conditions

The results of this study showed no significant difference in carotenoid degradation between sorghum stored at 4°C and 22°C, suggesting that storage temperature may not have a significant impact on carotenoid degradation. These results are inconsistent with previous studies that have shown that storage temperature has as a significantly effect on carotenoid degradation (Ceron- Garcia et al., 2010; Ortiz et al., 2016). Wet cooking methods, such as boiling, have been shown to cause significant carotenoid degradation in various crops, including sorghum (Berni et al., 2015; Taylor et al., 2015). However, the results of this study showed no significant difference in carotenoid degradation between the two wet cooking methods (couscous and porridge). These results are inconsistent with previous studies that have shown significant differences in carotenoid degradation between different cooking methods (Berni et al., 2015; Taylor et al., 2015). The differences in results may be due to variations in cooking time, temperature, and method. It can be concluded that the concentration of carotenoids in biofortified sorghum grains is affected by genotype, but not consistently affected by storage temperature, cooking style, or the interactions between these factors. In general, zeaxanthin, lutein, and β-carotene

concentrations were found to be significantly affected by genotype, while only zeaxanthin concentration was affected by cooking style, specifically by the interaction effect between cooking style and genotype. These findings are consistent with previous studies that have also found genotype to be a significant factor affecting carotenoid concentrations in sorghum grains (Olayinka et al., 2011; Liu et al., 2019). However, the effects of storage and cooking on carotenoid concentrations in sorghum grains have been inconsistent across studies, with some studies finding significant effects and others finding no significant effects (Olayinka et al., 2011; Tadesse et al., 2019, Dias et al., 2014). It is important to note that while storage temperature and cooking style may not consistently affect carotenoid concentrations in sorghum grains, they can still impact the overall nutritional value and bioavailability of these carotenoids. High temperatures and prolonged cooking times can lead to the breakdown of carotenoids and decrease their bioavailability (Maiani et al., 2009). For instance, a study conducted on the effect of processing on carotenoid degradation in sweet potatoes found that the most significant degradation occurred during storage, while minimal degradation was observed during cooking (Vimala et al., 2019). This is consistent with our findings in sorghum, where the most significant degradation occurred between pre-harvest and earlier time points. While the results of this study suggest that storage temperature and wet cooking styles do not significantly affect the concentration of carotenoids in biofortified sorghum grain, it is important to consider the limitations of the study. One possible reason we did not see significant effects of storage temperature and cooking style on carotenoid concentration could be due to the short duration of the study. Previous studies have reported that carotenoid degradation can occur over a longer period of time and may require extended storage and processing times to observe significant changes in concentration (Trono, 2019; Atencio et al., 2022). Another possible reason for the lack of significant effect could be the specific storage and cooking conditions used in this study. For example, the storage temperature range of 4°C to 22°C may not have been wide enough to observe significant differences in carotenoid degradation, as other studies have reported greater degradation at higher temperatures (Saleh et al., 2013; Chandrasekara et al., 2012). Similarly, the two wet cooking styles used in this study may not have been varied enough to fully capture the impact of different cooking methods on carotenoid concentration.

Other studies have reported significant differences in carotenoid degradation between different cooking methods, such as boiling, steaming, and frying (Saleh et al., 2013; Jiang et al., 2022, Zhang et al., 2019). Therefore, while this study provides valuable information on the stability of carotenoids in biofortified sorghum grain during storage and processing, further research is needed to fully understand the impact of storage and processing conditions on carotenoid concentration. Additionally, considering the potential impact of genotype on carotenoid concentration, future studies may benefit from evaluating a wider range of accessions to better inform storage and processing recommendations for biofortified sorghum grain. Most importantly this study showed significant degradation of carotenoids during post-harvest handling and processing. These findings have important implications for improving the nutritional quality of sorghum and setting accurate target concentrations for the biofortification program. These results can also be developed further to find the optimal storage and cooking methods to preserve the nutritional content of vitamin A biofortified grains.

Conclusion

These findings support the initial hypothesis that sorghum grain carotenoids will have an overall decrease in content from harvest to the final cooked product. Additionally, understanding the extent of carotenoid degradation during the post-harvest process is critical for determining the appropriate concentration of biofortified sorghum grain required to achieve optimal nutritional impact in populations with vitamin A deficiency. This information is also important for the commercialization of biofortified sorghum, as it can inform the development of effective storage and distribution strategies that maintain carotenoid content and preserve nutritional quality. Ultimately, continued research into the post-harvest processing of biofortified sorghum grain can contribute to improving the nutritional status of vulnerable populations and reducing the burden of vitamin A deficiency worldwide.

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APPENDIX

Supplementary Table 1: A candidate gene list, primer sequences, and efficiencies

Gene	Gene Identifier	Name	Location	Direction	Length	Sequence	Primer Efficiency
DXS Precursor	Sobic.002G064500	1-deoxy-D-xylulose 5-phosphate synthase	Chr02:6278447	FWD	22	AAG AGA TGA GAT GAA GGC AAG G	103%
				REV	22	GAG GAA TCA AGC AAG GAG TAC A	
DXR Precursor	Sobic.003G103300	1-deoxy-D-xylulose 5-phosphate reductoisomerase	Chr03:9224675	FWD	20	GCC CTG TCC CTG CAT AAT AA	109%
				REV	21	GGA CTG TGG AGG CAT GAT TTA	
GGPPS Precursor	Sobic.004G287300	HETERODIMERIC GERANYLGERANYL PYROPHOSPHATE SYNTHASE SMALL SUBUNIT	Chr04:62947408	FWD	20	CAA AGC CCA AAC CAA ACT CC	102%
				REV	19	CCA TCT CGC CGA ACT TCT T	
PSY#1 Biosynthesis	Sobic.002G292600	Phytoene synthase	Chr10:60961989	FWD	21	GCC AAA CGC CAA CTA CAT TAC	98%
				REV	22	GTC ACT CCT CAT CCC TTC AAT C	
PSY#2 Biosynthesis	Sobic.008G180800	15-cis-phytoene synthase	Chr08:61421399	FWD	21	GAC GGT CCT AAC GCA TCT TAC	104%
				REV	21	TCC AGC CTC ATT CCT TCA ATC	

PSY#3 Biosynthesis	Sobic.010G276400	15-cis-phytoene synthase	Chr02:67031666	FWD	19	ACG AGT GGA GGA GCT TCA T	107%
				REV	22	CCT CCT GGT GAA GTT GTT GTA G	
PDS Biosynthesis	Sobic.002G383400	15-cis-phytoene disasterase (Os07g0626800 protein)	Chr02:73907620	FWD	21	CCT TGT GCT GAT GTC CAG TAT	105%
				REV	20	GAG CTC GTC CAT GAA CTT GT	
βCH Degradation	Sobic.006G188200	B-carotene hydroxylase	Chr06:54257421	FWD	22	ACA TAA TGG AGG CCG TCT AAT C	106%
				REV	22	CCG CGA CTG ATC TCT TCT ATA C	
ZEP Degradation	Sobic.006G097500	zeaxanthin epoxidase	Chr06:46719605	FWD	20	CAG ATG TCG GTG CTG GTA AA	105%
				REV	22	CGT ATA TAT CCC GGC GAA GAA C	
AAO Degradation	Sobic.002G225400	Abscisic acid 8'-hydroxylase 3	Chr02:61737120	FWD	23	CCT CCT AGC CAC CAA CAT ATA AA	101%
				REV	21	CCT GCA TCG CCT TGT AAT AGA	
EIF4A Reference	Sobic.010G251100.1	EUKARYOTIC INITIATION FACTOR 4A-1-RELATED	Chr10:59036897	FWD	21	GGT GAC TAC CTG GGT GTT AAG	102%
				REV	20	GGC TGG AAG AAG TTG GAA GA	
PP2A Reference	Sobic.004G092500.1	Serine/threonine-protein phosphatase PP2A-5 catalytic subunit	Chr04:7869694	FWD	21	GTC TTC AGT GCG CCT AAC TAT	104%
				REV	21	GAG GAG CAT CAA CAC CAG TAA	

Supplementary Table 2: Average Concentration of Lutein, Zeaxanthin, and B- Carotene in Grain, Leaf, and Root Tissue for all Accessions

Genotype	Tissue	Carotenoid	Concentration (ug/g)
510951	Grain	Lutein	0.58748
511015	Grain	Lutein	0.65925
511018	Grain	Lutein	0.78727
585347	Grain	Lutein	1.63036
585348	Grain	Lutein	4.04501
585369	Grain	Lutein	2.01665
510951	Leaf	Lutein	362.45951
511015	Leaf	Lutein	309.71699
511018	Leaf	Lutein	236.86021
585347	Leaf	Lutein	373.24994
585348	Leaf	Lutein	323.66104
585369	Leaf	Lutein	261.2863
511015	Root	Lutein	0.45732
511018	Root	Lutein	0.55864
585348	Root	Lutein	0.45272
585369	Root	Lutein	0.54442
510951	Grain	Zeaxanthin	0.35697
511015	Grain	Zeaxanthin	0.37292
511018	Grain	Zeaxanthin	0.68585
585347	Grain	Zeaxanthin	1.3449
585348	Grain	Zeaxanthin	2.25846
585369	Grain	Zeaxanthin	1.48795
510951	Leaf	Zeaxanthin	6.15229
511015	Leaf	Zeaxanthin	6.89831
511018	Leaf	Zeaxanthin	6.43901
585347	Leaf	Zeaxanthin	5.73059
585348	Leaf	Zeaxanthin	5.75389
585369	Leaf	Zeaxanthin	10.85959
511018	Root	Zeaxanthin	0.36918
585347	Root	Zeaxanthin	0.54922
585348	Root	Zeaxanthin	0.5137
510951	Grain	β carotene	0.28915
511015	Grain	β carotene	0.33865
511018	Grain	β carotene	0.30629
585347	Grain	β carotene	0.69855
585348	Grain	β carotene	1.31691
585369	Grain	β carotene	0.92506
510951	Leaf	β carotene	270.08483
511015	Leaf	β carotene	159.27658
511018	Leaf	β carotene	67.20937
585347	Leaf	β carotene	196.46683
585348	Leaf	β carotene	150.54311
585369	Leaf	β carotene	79.95072

510951	Root	β carotene	0.49876
511015	Root	β carotene	0.60877
511018	Root	β carotene	0.52754
585347	Root	β carotene	1.06619
585348	Root	β carotene	1.02638
585369	Root	β carotene	0.78402