GENETIC ALTERATION OF PLANT SECONDARY METABOLISM:
MODIFICATION, ENHANCEMENT AND
CHARACTERIZATION OF PIGMENTS IN TOMATO (LYCOPERSICON
ESCOLENTUM) FRUIT

A Thesis

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ABSTRACT

Carotenoids and other plant-associated pigments represent a rich source of dietary nutrients and other potentially bioactive compounds. A correlation between the intake of carotenoids and the reduction of cancer and heart disease has been noted. The antioxidant effects of lycopene suggest a possible mechanism of activity but a causal relationship remains to be demonstrated. Epidemiological evidence that suggests a role for lycopene in reducing risk of disease has generated interest in this carotenoid as a food with function that includes health promoting properties as well as dietary needs. There are limitations in the use of carotenoids as so called functional foods because the effect of their structure and concentration on uptake and biological activity in the human body is not understood. The objectives of this study were to: 1) Develop tomato lines with enhanced concentration and altered structure of carotenoids and determine the importance of isomeric structure of lycopene on cyclization reactions in carotenoid biosynthesis; 2) Apply HPLC metabolic profiling to evaluate the secondary metabolites of the tomatoes developed under objective 1; 3) Study the phytochemistry of “purple heirloom” tomatoes as a potential source of increased dietary pigments. We used molecular-marker-assisted and classical selection to combine genes that affect both the biochemical synthesis of carotenoids and the density of the chromoplast to develop tomato lines varying in
carotenoid concentration and structure. To test the effect of genetic background on carotenoid profile, tomatoes segregating for tangerine \((t)\) and ripening inhibitor \((rin)\) genes were crossed to a tomato line carrying the tangerine virescent \((t^v)\) allele. Populations were developed by crossing the heirloom varieties to processing tomato breeding lines, followed by self fertilization to develop segregating populations for selections. Two hypotheses, increased poly-phenols and retention of chlorophyll, were tested regarding the basis of pigmentation in these varieties described as “black” and “purple” were tested.

We developed tomato lines with improved and altered concentration of carotenoids. Selected lines ranged in concentration from 0-132 \(\mu g/gm\) lycopene, 0-52 \(\mu g/gm\) \(\beta\)-carotene, 0-45 \(\mu g/gm\) \(\delta\)-carotene, 0-52 \(\mu g/gm\) tetra-\emph{cis} lycopene, and 0-62 \(\mu g/gm\) \(\zeta\)-carotene on a fresh weight basis. When dark green \((dg)\) was combined with \(t\) and \(t^v\) the concentration of carotenoid precursors was also altered. Combination of the gene \emph{Delta} with \(t\) and \(t^v\) suggest that lycopene \emph{\(e\)-cyclase} does not efficiently use tetra-\emph{cis} lycopene as a substrate and to convert it to \(\delta\) –carotene. These results emphasize the important role of genetic interactions on the concentration and structure of carotenoids in tomato fruit.

The presence of the \emph{rin} mutation had a very significant effect on the carotenoid profile. When \emph{rin} was present in the homozygous condition, the concentration of tetra-\emph{cis} lycopene decreased by 91%. There was a 45% decrease in tetra-\emph{cis} lycopene when \emph{rin} was in the heterozygous condition, demonstrating that hybrids employing the \emph{rin} locus to impart long shelf life may have a lower level of certain carotenoids. However, an increase
in β-carotene was observed when rin was present in both the homozygous and heterozygous condition in the tangerine background, with the homozygous rin lines having significantly more β-carotene than the heterozygous. No ζ-carotene and neurosporene were detected when rin was present in the homozygous condition, and these compounds decreased 42% and 49%, respectively, relative to wild type plants when rin was in the heterozygous condition. When rin was in the homozygous and heterozygous condition, phytoene decreased 88% and 51%, respectively, relative to wild type plants. The allele present at the tangerine locus did not have a significant effect on the profile of carotenoid precursors, but the concentration of tetra-cis lycopene and β-carotene did vary, with lines carrying the t allele containing more β-carotene and lines carrying the t' allele containing more tetra-cis lycopene.

Segregating populations developed by crossing purple heirloom varieties to processing tomato breeding lines segregated for a range of pigment types and colors. HPLC traces showed that populations ranged in lycopene concentration from 11-108 μg/g, β-carotene from 3-7 μg/g, and tetra-cis lycopene concentration from 6-237 μg/g on a fresh weight basis. An assay of total phenolics demonstrated that ‘Black Plumb’ had significantly higher amounts of poly-phenol compounds compared to the other parents used to develop this population. Varieties ‘Black’ and ‘Black Prince’ did not differ in poly-phenol content from breeding lines Ohio 02-1023-1 and Ohio 02-1025-1. The heirloom varieties tested maintained chlorophyll after ripening. Allelism test-crosses between purple heirloom varieties and genetic stocks carrying the green flesh (gf) gene resulted in hybrids having the purple phenotype. These results suggest that the gf
mutation is responsible for the purple phenotype, and that the purple color in the heirloom varieties we studied is due to the retention of chlorophyll as the plastid accumulates lycopene. The germplasm developed will be of further use in research to address questions concerning the bioavailability and bioactivity of phytonutrients using \textit{in vitro} and \textit{in vivo} models.
Dedicated to my parents for their guidance and love
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CHAPTER 1

LITERATURE REVIEW

Introduction

Tomato is an important crop worldwide. This fruit, consumed as vegetable, is the second most produced and consumed vegetable crop in North America with a per capita consumption of 19 pounds (anonymous). Although tomato fruits are often consumed fresh, it is estimated that 80% of the consumed tomatoes are from tomato products such as juice, ketchup, soup, sauce, puree, and paste (Gould, 1992). Additionally, health benefits have been linked to diets rich in tomatoes and tomato products. The combination of the important economic demand of tomatoes and the potential health benefits make tomato fruits an important target for increasing the nutritional content.

Clinical nutrition studies have suggested a correlation between increased consumption of red tomato products and a reduced risk of developing certain cancers (Gann et al., 1999; Giovannucci et al., 1995; Giovannucci, 1999; Sies and Krinsky, 1995). The antioxidant effects of lycopene suggest a possible mechanism of activity (Clinton, 1998; Nguyen and Schwartz, 1999), but a causal relationship remains to be demonstrated. It has been difficult to separate the effect of dietary carotenoids in studies involving tomato products due to the lack of detailed characterization of varieties with
significant differences in these compounds. In addition, there is a lack of knowledge concerning how the structure and concentration of key dietary carotenoids affects uptake and biological activity in the human body, limiting the utilization of these compounds as functional foods or medicines. For example, lycopene (a carotenoid) exists in tomatoes as a trans-isomer, but cis-isomers are detected in human plasma following consumption. When lycopene is ingested in its natural trans form, it is poorly absorbed while its cis isomer is more readily absorbed (Unlu et al., 2003).

The aim of the present work was to develop tomatoes that vary in the concentration of trans-lycopene and tetra-cis lycopene in order to test specific hypotheses about the effect of concentration, steric structure, and competition on antioxidant activity and nutritional availability. These lines allowed us to test the role of steric hindrance on the efficiency of cyclization reactions during carotenoid biosynthesis. In addition, two competing hypotheses that explain the purple color of heirloom tomatoes were also tested. Developing tomato lines that vary in pigment content and concentration will provide a source of raw material for human health research.

The objectives of this work were:

1) Develop tomato lines with enhanced concentration and altered structure of carotenoids and determine the importance of isomeric structure of lycopene on cyclization reactions during carotenoid biosynthesis.

2) Apply HPLC metabolic profiling to evaluate the secondary metabolites of the tomatoes developed under objective. 1
3) Study the phytochemistry of “purple heirloom” tomatoes as a potential source of increased dietary pigments.

Carotenoids

Carotenoids are pigments derived from phytoene. They are of lipid nature and are yellow to red in color. Their structure is based on a tetraterpene skeleton, which can be cyclized at one end or both ends of the molecule (Packer et al., 1999). Cyclization at one end of the molecule of lycopene yields δ-carotene and γ-carotene. Cyclization at both ends produces β-carotene and α-carotene. One of the common features of carotenoids is their extended chain with conjugated double bonds, which are considered to be responsible for both their antioxidant activity and color (Stahl and Seis, 1999). The most abundant carotenoid in tomato, lycopene, predominantly exists in the trans form, but cis isomers are also encountered in tomato varieties carrying the tangerine mutation.

Carotenoid isomerization in tomato was explained by studying alleles of the tomato gene tangerine (t) (Isaacson et al. 2002). The enzyme coded by the wild type tangerine gene, carotenoid isomerase (CRTISO), has a structure related to the bacterial-type phytoene desaturase (crtl). The CRTISO enzyme converts tetra-cis-lycopene to all-trans-lycopene (Figure 1.1). Fruits of tangerine tomatoes lack a functional CRTISO leading to the accumulation of tetra-cis lycopene at the expense of all trans lycopene. The reason for the accumulation of tetra-cis lycopene when CRTISO is absent is still unclear but Isaacson et al. (2002) hypothesized that lycopene cyclases cannot cyclize tetra-cis lycopene because of steric hindrance. Mutants at tangerine yield either a nonfunctional or
an altered CRTISO. The mutation has been characterized for several alleles. A deletion in the first exon of the $t^{mic}$ allele and a deletion in the promoter in $t^{3183}$ allele, which seems to alter its expression in the fruit, are two samples (Isaacson et al. 2002). The molecular basis for a third allele, $i^{v}$ (tangerine virescent), is not known.

Figure 1.1. The enzyme CRTISO catalyzes the formation of all-trans-lycopene from tetra-cis-lycopene.
Other mutations such as *Delta, Beta, old gold (og)*, and *old gold crimson (og*) affect carotenoid biosynthesis in tomato. The *Delta* mutation is dominant and enhances the activity and expression of lycopene ε-cyclase (Ronen et al., 1999). *Delta* mutants produce orange-colored fruits when in homozygous and heterozygous state. The *Beta* mutation is partially dominant and the orange-colored fruit is caused by the enhanced transcription of lycopene β-cyclase and the resulting hyper-accumulation of β-carotene (pro-vitamin A). The *og* and *og*\(^e\) mutations are recessive alleles of *Beta* and fruits are deep red, lacking β-carotene due to a decrease of fruit specific lycopene β-cyclase activity (Ronen et al, 2000). Crosses with *og* and *og*\(^e\) increase lycopene at the expense of β-carotene.

Genes available for the manipulation of carotenoid concentration include those that affect the chromoplast. The high pigment genes (*hp*-1, *hp*-2 and its allele *dark green* (*dg*)), and the *green flesh* (*gf*) gene affect the number of chromoplasts or the transition from chloroplast to chromoplast. Therefore these genes affect pigmentation through a distinct mechanism relative to the biosynthetic enzymes.

**Biological Activity of Carotenoids.**

In many physiological processes, reactive oxygen species (ROS) are produced and are able to harm important molecules such as lipids, carbohydrates, proteins and nucleic acids. The biological system is thought to be protected by carotenoids through physical quenching of singlet oxygen (\(^1\text{O}_2\)). In this process, the exited \(^1\text{O}_2\) transfers its energy to the carotenoid molecule. Lycopene, being highly unsaturated and containing a
straight open-chain and 11 conjugated and 2 non-conjugated double bonds, represents an efficient singlet oxygen quencher (Stahl and Seis, 1999). Its effectiveness as an antioxidant suggest a possible mechanism for protection of cells against oxidative damage. Due to these potential properties, lycopene is considered a pigment of commercial importance in the nutraceutical market.

Studies in the medical field have linked carotenoids to biological activity. Research on the inhibition of human promyelocytic leukemia cell growth by acyclic carotenoids, phytofluene, ζ-carotene and the oxidation mixture of lycopene, demonstrated that these carotenoids significantly inhibited the cell growth inducing apoptosis in HL-60 cells, with ζ-carotene being the most efficient (Nara et al., 2001). Other studies suggest that carotenoids also function as modulators in the immune system, and gene activators (Setiawan et al., 2000). Carotenoids such as β-carotene are of dietary significance due to their pro-vitamin A activity (Mayne, 1996). A scarcity of vitamin A can lead to blindness while complete deficiency can lead to premature embryonic death (Kiefer et al., 2002). There remains a high incidence of vitamin A deficiency in developing countries, with preschool children and women of reproductive age being the most affected (WHO/UNICEF, 1995). Likewise, some studies have also indicated that following a diet with food rich in carotenoids will diminish risk of developing certain cancers and chronic diseases (Hedren et al., 2002).

Lycopene has high demand not only in the pharmaceutical industries but also in the food and cosmetic industries. This demand has lead to interest in the development and production of plants with high lycopene content for the industrial production of high
lycopene products (Gomez-Prieto et al., 2003). Lycopene is not widely available; it exists in a very limited number of fruits and vegetables. The major source of lycopene in North America is tomato and tomato products. Developing tomato lines with high concentration of lycopene in the cis and trans form may be a potential source of material for human health research.

**Flavonoids**

Flavonoids are water-soluble poly-phenol derivatives, which accumulate in the epidermal tissue of plant organs where there is a high UV-B radiation exposure, serving as protection against radiation tissue damage (Saslowsky and Winkel-Shirley, 2001). Flavonoids are normally found in the vacuoles, chromoplasts and chloroplasts. They represent a very diverse group of compounds of low molecular weight having in common the basic structural unit the C\textsubscript{15} skeleton. Within the group of compounds commonly referred to as poly-phenols, the flavonoids are classified as polycyclic aglycons. The basic structure of their aglycones consists of two aromatic rings united by a 3-carbon chain (Figure 1.3).
Figure 1.2. Carotenoid biosynthetic pathway. Enzymes are named according to their function. Phytoene desaturase (Pds); phytoene desaturase (crtl); \( \zeta \)-carotene desaturase (Zds); carotenoid isomerase (CRTISO); lycopene \( \beta \)-cyclase (CrtL-b); and lycopene \( \varepsilon \)-cyclase (CrtL-e). \( ^1 \)crtl is the bacterial type phytoene desaturase which converts phytoene to trans-lycopene.
Flavonoids are classified according to the structure of the C₃ central chain (Goodwin and Mercer, 1983). Based on this system, flavonoids are classified as chalcones, flavanons, flavanols, flavones, isoflavones, catechins and anthocyanidins as shown in Figure 1.4. Within each family of phenols, many compounds may exist and more than 4000 different flavonoids occurring in plants have been described (Hollman, 2001).

Flavonoids are biosynthesized from the shikimic acid pathway (ring B, which sometimes has hydroxyl substitution at C-3', C-4' and C-5' positions) and the Acetyl CoA pathway via Malonylcoenzyme A (ring A, which is commonly hydroxylated at the positions C-5 and C-7). The biosynthesis of flavonoids occurs in the cytosol. The first C-15 flavonoid, which is a chalcone, arises from the reaction of 4-Coumaroyl CoA and Malonyl CoA. This reaction is the first committed step in the flavonoid metabolism.

Genes encoding enzymes in the flavonoid biosynthetic pathway that have been cloned and characterized include: chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), flavonol synthase (FLS), dihydroflavonol reductase (DFR), leucoanthocyanidin dioxygenase (LDOX)
(Burbulis and Winkel-shirley, 1999), glucosyl transferase, and rhamnosyl transferase (Verhoeven et al., 2002). The biosynthetic pathway for the most important flavonoids in tomatoes is summarized in Figure 1.5.

Flavonoids are secondary metabolites that are important in many plant processes (Bovy et al., 2002). Processes affected by flavonoids include pigmentation, resistance to pathogen attack, seed development, pollen growth, response to nutrient deprivation, and UV light protection. They also function as allelopathic compounds, which inhibit the growth of plants that are nearby. Flavonoids also mediate both beneficial and pathogenic host-microbe interactions through the induction of microbial genes such as the \textit{vir} genes in \textit{Agrobacterium} and the \textit{nod} genes in \textit{Rhizobium} (Spencer and Towers, 1988; Peters et al., 1986; Maxwell et al., 1990).

As a dietary component, flavonoids, in particular flavonols (kaempferol and quercetin), have been suggested to have health-promoting properties because of their high antioxidant capacity (Pietta 2000). Epidemiological research suggests a direct correlation between high flavonoid intake and the reduction of risk of heart disease, reduced inflammation, the induction of detoxifying enzyme systems, and the inhibition of enzymes involved in tumorigenesis (Miean and Mohamed, 2001). A study conducted by Wang et al. (2003) supported the theory that flavonoids are associated with the reduction of cancer risk by inhibiting the insulin-like growth factor-I (IGF-I) signaling cascades. The antioxidant activity of these compounds is due to their reactive hydroxyl group, which oxidizes free radicals to a more stable and less reactive form. The chemistry of
their antioxidant activity suggests a mechanism through which they may protect proteins, lipids and DNA in humans from radical damage (Howard et al., 2002).

Figure 1.4. Flavonoid classification.

Such studies have increased interest in enhancing the flavonoid content of foods using genetic modification to provide raw material with potent antioxidant activity for improving human health. Tomato represents an excellent candidate because it is one of the most important food crops worldwide and the peel of its fruit already contains modest levels of flavonols. The major flavonoids in tomatoes are kaempferol, quercetin, and
rutin. The anthocyanin petunidin has been reported in the tomato carrying the

*Anthocyanin fruit (Aft)* gene (Jones et al., 2003). Thus, like carotenoid modification, genetic approaches can be used to enhance and alter flavonoid content in tomato fruit.

**Chlorophyll: its biosynthesis and degradation**

In nature, chlorophylls are the most abundant pigment and they comprise as much as 1% of the dry weight of certain tissues (Ferruzzi et al., 2001). Chlorophyll refers to a family of compounds, which are magnesium complexes of tetrapyrrole derivatives. These substances are of green color. The main function of chlorophylls is to absorb solar radiation and transform it into chemical energy. They constitute the most important group of pigments for photosynthesis.
Figure 1.5. The biosynthetic pathway for the most important flavonoids in tomato, kaempherol, quercetin, and rutin.
The molecule of chlorophyll is composed by a porphyrin head which has a central magnesium atom bonded to the four nitrogen atoms of the pyrrole rings (Figure 1.7). The chlorophyll molecule also contains a long carbon chain (C\textsubscript{20}H\textsubscript{39}OH). This carbon chain possesses hydrocarbon radicals and due to these hydrocarbon radicals, the chlorophyll molecule is lipid soluble (Vazquez and Torres, 1995). Eight different chlorophylls have been identified. There are four main compounds (a, b, c and d) in two groups, chlorophyll and bacteriochlorophyll. Chlorophylls a and b are found in green plants, while chlorophylls c and d are restricted to brown algae. In the bacteriochlorophyll, a and b are present in photosynthetic bacteria, while c and d (or chlorobium chlorophylls) occur in the Chlorobacteriaceae (Hendry et al., 1987). Chlorophyll a often co-exists with chlorophyll b, with chlorophyll a being the most abundant. Chlorophyll a ranges in color form green to blue, and chlorophyll b from green to yellow (Vazquez and Torres, 1995). With few exceptions, chlorophylls are dihydroporphyrins in which one of the four pyrrole
rings has been reduced (Figure 1.6). The bacteriochlorophylls are based on the tetrahydroporphyrin structure. In this group two of the pyrrole rings have been reduced (Hendry et al., 1987).

![Chlorophyll](image)

Figure 1.7. Molecule of chlorophyll

The biosynthetic pathway of chlorophyll is well understood while knowledge of the degradation is still limited. The mechanism of chlorophyll degradation is important because this breakdown marks the chloroplast-chromoplast transition. This turnover is fundamental to the physiology and biochemistry of plants and it represents the largest flux of environmental carbon (Hendry et al., 1987). One of the main reasons for the poor understanding of chlorophyll degradation is that the genes that encode the enzymes mediating this pathway are just starting to be identified. Takamiya et al., 2000, proposed a pathway for chlorophyll degradation, which comprises two stages and five enzymes.
(Figure 1.8). In the first stage the products are still green while in the late stage they are colorless. Ni et al., 2001 proposed an alternative pathway for the degradation process, which involves three enzymes in two pathways: the pheophorbide pathway and the oxidative bleaching pathway. According to Tsuchiya et al., (1999), the early steps of chlorophyll degradation take place in the chloroplasts, then the fluorescent catabolites of chlorophyll are exported from chloroplasts to the cytosol where they are somewhat modified and then imported into the vacuole.

Carotenoids and chlorophylls are associated in all photosynthetic tissue. The differentiation of chloroplasts into chromoplasts is marked by the breakdown of chlorophyll and the massive synthesis of carotenoids (Lawrence et al., 1997). The green flesh (gf) gene alters this normal chloroplast-chromoplast transition (Cheung et al., 1993). In the gf mutant of tomato, chlorophyll, thylakoids and some thylakoid proteins are retained even as the plastid accumulates lycopene in the transition to chromoplast (Akhtar et al., 1999).

Heirloom tomatoes with flesh color described as "purple" are a potential source of dietary pigments, but these varieties have not been subjected to careful analysis. It is possible that high concentration of flavonoids could impact color as in lines carrying the Aft gene. The genetic control of poly-phenols in tomato fruit is poorly understood, though poly-phenols such as kaempferol, quercetin, rutin, and sometimes anthocyanins are found in the skin (Bovy et al., 2002; Jones et al., 2003). Alternatively, a superimposition of green chlorophyll and red lycopene may result in a purple color (as is seen in the gf mutation). Despite competing hypothesis explaining their color, “purple
heirloom” tomatoes may provide a source of increased dietary pigments resulting from the attenuated degradation of chlorophyll or increased flavonoids.

Figure 1.8. Degradation pathway of chlorophyll propose by Takamiya et al. (2000). In the early stage the catabolites are still green while in the late stage they are colorless. “RCC, red chlorophyll catabolites; pFCC, primary fluorescent chlorophyll catabolites; NCC, non fluorescent chlorophyll catabolites”.
SUMMARY

Cancer and heart diseases are the two primary causes of death in the United States. Both diseases have been linked to oxidative damage (Nelson et al., 2003, Rao et al., 2000). Mortality due to cancer accounts for one out of four deaths, and this rate is only exceeded by heart disease. Clinical studies have shown a correlation between increase consumption of red tomato and tomato products, and decrease of the risk of developing certain cancers. (Clinton, 1998; Nguyen and Schwartz, 1999). Other studies suggest that following a diet with food rich in carotenoids reduces the risk of developing certain cancers, chronic diseases, macular degeneration and radiation damage (Hedrén et al., 2002; Fraser et al., 2001; Van Breemen et al., 2002). Lycopene, the most studied carotenoid, mainly exists in the trans configuration while in the human plasma it exists as an isomeric mixture (van Breemen et al., 2002; Stahl and Sies, 1996). However it has not been reported how the structure and concentration of carotenoids affect the biological activity and uptake in the human body although studies have reported that when lycopene is in the cis configuration its bioavailability increases as well as it absorption (Unlu et al., 2003).

Several naturally occurring alleles of genes affect carotenoid content and structure. Tomatoes with lycopene in the cis configuration are found in varieties carrying
the *tangerine* mutation. This mutation produces a non-functional carotenoid isomerase (CRTISO) leading to the accumulation of tetra-cis lycopene instead of *trans*-lycopene. In the carotenoid biosynthesis, CRTISO is the enzyme that converts tetra-cis lycopene to *trans*-lycopene (Isaacson et al., 2002). Other mutations found in tomatoes that affect the carotenoid biosynthesis pathway include the *Delta*, *Beta*, and *og* mutations. In addition to those mutations, others affect the chromoplast structure (*gf*) while others affect the chromoplast density (*dg*).

Through the manipulation of several major genes there is a great potential to increase concentration, and alter structure of carotenoids in tomato fruits. Tomato represents an excellent model to manipulate pigments in fruits since very diverse germplasm and molecular tools exist, and it is one of the major crops in North America (Vrebalov et al., 2002). The present study used classical and molecular assisted techniques to develop tomato lines with altered concentration and structure of carotenoids, and applied HPLC profiling to evaluate their secondary metabolites.
REFERENCES


Anonymous. URL: www.tomato.org


CHAPTER 2

Plant genomic resources for testing physiological effects of dietary carotenoids.

ABSTRACT

Correlative evidence suggests that enhanced consumption of carotenoids may decrease risk of cancer. Lack of knowledge concerning how carotenoid structure and concentration affect uptake and biological activity in the human body limits the use of these compounds as functional foods. Further, variation in the concentration and isomeric structure of specific carotenoids exists within varieties of tomato, and modification of one carotenoid often affects related compounds in the biochemical pathway. For example, increased content of lycopene is often accompanied by a decrease in β-carotene, thus it is difficult to separate the effects of one carotenoid from another. In order to develop genetic resources to test the physiological effects of dietary carotenoids in the food matrix, we used molecular-marker-assisted and classical selection to combine genes that affect both the biochemical synthesis of carotenoids and the structure of the chromoplast. We developed tomato lines with enhanced and altered concentration of
carotenoids. The lines range in concentration from 0-132 µg/gm lycopene, 0-52 µg/gm β-carotene, 0-45 µg/gm δ-carotene, 0-52 µg/gm tetra-cis lycopene, and 0-62 µg/gm ζ-carotene on a fresh weight basis. When genes that affect chromoplast quantity (dg) are combined with genes that affect the conversion of tetra-cis lycopene (t and t') to lycopene, ζ-carotene concentration is also altered. This result emphasizes the important role of genetic interactions on the concentration of carotenoids in the tomato fruit. A strategy to evaluate the physiological significance of bioactive components in the food matrix will involve blending product from specific lines in order to control both concentrations and ratios of the specific carotenoids under study as well as precursors that may impart biological activity.

INTRODUCTION

A correlation between the intake of carotenoids and the reduction of cancer and heart diseases has been documented (Clinton, 1998). The antioxidant properties of carotenoids suggests a possible mechanism of activity (Nguyen and Schwartz, 1998). Research on the inhibition of the growth of human promyelocytic leukemia cells by acyclic carotenoids, demonstrated that phytofluene, ζ-carotene and lycopene isomers significantly inhibited cell growth and induced apoptosis in HL-60 cells (Nara et al., 2001). These results also showed that the effectiveness of these carotenoids was concentration dependant with higher concentrations more effective than lower concentrations. These findings further suggest a biological role for lycopene precursors,
with ζ-carotene having the most dramatic effects. Studies also suggest a function for carotenoids in modulating the immune system, and as activatores of genes (Setiawan et al., 2001). Finally, the carotenoid β-carotene has a clear dietary role as a vitamin A precursor.

Modification of one carotenoid often affects related compounds in the biochemical pathway. For example, increased content of lycopene may be accompanied by a decrease in β-carotene (Thompson, et al., 1965; Lee and Robinson, 1980). Thus there is a need to improve knowledge of how carotenoid structure and concentration affect uptake and biological activity in the human body so that the established role as a nutrient for β-carotene can be balanced with the potential role of precursors to reduce chronic disease.

Evidence that diets with food rich in carotenoids may reduce the risk of developing certain cancers and chronic diseases suggests the potential to develop foods that have a function beyond meeting basic human nutrient needs (Hedren et al., 2002). Tomato is an excellent candidate for the development of functional foods due to its role as the major source of dietary carotenoids in North America and the availability of naturally occurring genetic variants that affect carotenoid content and structure. However, there is a limited knowledge regarding how concentration, structure, and ratios of specific carotenoids affect activity and uptake in the human body. The trade off between lycopene and β-carotene suggests that increasing lycopene or lycopene precursors in functional foods may have undesirable negative consequences. There is a corresponding lack of knowledge concerning adsorption and utilization of carotenoid
compounds in the diet. For example, lycopene in tomato fruit exists predominantly in the
*trans* form, while in the human plasma lycopene is found as an isomeric mixture (van
Breemen et al., 2002; Stahl and Sies, 1996). When lycopene is ingested in the *trans* form,
it is poorly absorbed while its *cis* isomer is more readily absorbed (Unlu et al., 2003).
There is a need to develop plant genetic resources to further study the physiological role
of carotenoids in the human diet and as functional foods.

Several well characterized genes exist in tomato for the modification of fruit
pigment through traditional crossing and selection, making tomato an ideal model plant
for developing genetic resources for testing physiological function in the human diet.
The *Beta* gene, coding for a fruit specific β-cyclase, exists in an allelic series allowing
the development of varieties that have fruit that are either high in lycopene or β-carotene
relative to standard tomatoes. In the *Beta* allele, an over expression of the fruit-specific
lycopene β-cyclase leads to the accumulation of β-carotene at the expense of lycopene.
Alleles of *Beta*, *og* and *ogc*, have the opposite effect by coding for a non-functional β-
cyclase. In addition, tomato fruit with pigments in a *cis* configuration are encountered in
lines and varieties carrying the *tangerine* gene. Fruits of *tangerine* tomato varieties lack a
functional isomerase (CRTISO) leading to the accumulation of tetra-*cis* lycopene at the
expense of all-*trans* lycopene (Isaacson et al. 2002) and fruit of tangerine tomatoes have
an attractive orange color. The *tangerine* gene also exists as an allelic series, with the *tmic*
allele containing a deletion in the first exon of the CRTISO creating an early stop codon.
The *t3183* allele has a deletion in the promoter, which presumably alters its expression in
the fruit. The molecular basis for a third allele, *t*′ (*tangerine virescent*), is not known.
Other naturally occurring variants include the \textit{Delta} gene which results in an over expression of lycopene \(\varepsilon\)-cyclase and the accumulation of \(\delta\)-carotene at the expense of lycopene. The high pigment genes (\textit{hp-1}, \textit{hp-2} and its allele dark green \textit{dg}) affect the number of chloroplasts in green tissue and chromoplasts in fruit. These genes therefore affect pigmentation through a distinct mechanism relative to the biosynthetic enzymes. Crosses with the high pigment genes generally increase the total carotenoid concentration (Sacks and Francis, 2001).

Developing tomato lines that combine high chromoplast pigment concentration with genes that affect the structure and concentration of carotenoids may be a potential source of material for human health research. Blending product from specific lines, in order to control both concentrations and ratios of the specific carotenoids under study and their precursors, will offer an approach to estimate the physiological significance of bioactive components in the food matrix. The objectives of this study were to develop tomato lines with enhanced concentration and altered structure of carotenoids.

\textbf{MATERIALS AND METHODS}

\textit{Population design.} Tomatoes (\textit{Lycopersicon esculentum} Mill.) carrying combinations of genes and alleles affecting the biochemical synthesis of carotenoids and genes and alleles affecting the chromoplast were developed through crossing and selection. Naturally occurring alleles leading to the high lycopene phenotypes associated with the \textit{dark green} (\textit{dg}) allele of the \textit{hp-2} gene and the \textit{old gold crimson} (\textit{og}) allele of
the fruit specific β-cyclase have been described (Levin et al., 2003; Ronen et al., 2000).

The high lycopene tomato carrying both the dg allele and the og\(^c\) allele was FG99-218, which derives from T4099 (Wann, 1997) back-crossed to Ohio 9242. Lines carrying the tangerine gene were crossed with lines carrying dg and og\(^c\) to select tomatoes with varying levels of tetra-cis lycopene. Two alleles of tangerine were used in population development. The variety Carolina Gold (a gift of Randy Gardner, North Carolina State University) was the source of the \(t\) allele and the inbred line Ohio 02-1023 was the source for the \(t'\) allele introduced from the genetic stock LA0351 into Ohio 9242. Inbred lines and varieties carrying either the Delta gene or the Beta gene were crossed with FG99-218 to select tomatoes with elevated levels of δ-carotene and β-carotene respectively. The source of the Delta allele was LA716, crossed into the variety M82 (Eshed and Zamir, 1995); and the source of the Beta allele was the USDA bred variety 97L97 which contains Beta from the wild tomato Lycopersicon cheesmanii, accession LA0317, crossed into Ohio 8245 (Stommel, 2001). To address steric hindrance during cyclization reactions, crosses between tomatoes carrying the tangerine gene (\(t'\) and \(t\)) and lines carrying Delta were made.

**Experimental design.** The experimental design for this study included two phases, a selection phase in which tomatoes with specific gene combinations were selected, and a testing phase in which lines were replicated and measurements of carotenoid pigments were made. Selection was based on marker-assisted selection (MAS) and classical methodologies as described below. F\(_2\) plants were selected in the
greenhouse and transplanted directly to the field where genotypes with specific combinations of alleles were the unit of replication.

**Greenhouse and field growth conditions.** Segregating populations (F2) were seeded into 288-well flats containing Metro Mix 360 soil media (Premier Horticulture Inc., Red Hill, PA). Plants were grown in a greenhouse with a day temperature of 23.8°C ±3°C and a night temperature of 18.3°C ±3°C with a 14/10 h light/dark period. Supplemental high-intensity discharge lighting was provided when natural light fell below 200 W/m². Seedlings were fertilized twice per day with 20 ppm N-P-K (Peters Professional All-Purpose Fertilizer, 20-20-20, Scotts-Sierra Horticultural Products Co., Marysville, OH). Selected F2 plants were planted in the field at the Horticulture and Crop Science Farm, Wooster, OH following the procedures described in the Ohio Vegetable Production Guide (Precheur, 2000). They were placed in plots containing 5 to 10 plants, with 30 cm spacing between plants within rows, and 1.47 meter spacing between rows. All genotypes were replicated in two randomized complete blocks.

**Genomic DNA extraction for genetic analysis.** Genomic DNA from parental lines was extracted from single leaflets. Each leaflet was ground in a polyethylene sample bag (0.004 gauge) with 1 ml of extraction buffer (0.35 M Sorbitol, 0.1 M Tris, 0.005 M EDTA, pH 7.5, 3.8 g/l NaBisulfite added just before use) by gentle rubbing. 300 µl of the extraction were transferred to a 1.5 ml tube, 300 µl of lysis buffer (0.2 M Tris, 0.05 M EDTA, 2.0 M NaCl, 2% CTAB, pH 7.5) and 150 µl of 5% sarcosine were added. Samples were incubated at 65°C for 15 min. After the incubation period, 700 µl of chloroform: isoamyl alcohol (24:1) were added and centrifuged for 15 min at 13800 g.
The aqueous phase was removed to a fresh 1.5 ml tube and the chloroform:isoamyl alcohol extraction was repeated one more time. DNA was precipitated by addition of 0.8 volume of isopropanol followed by centrifugation at 13800 g for 15-20 min. After centrifugation, precipitated DNA was dried and resuspended in 500 µl of T1/10 E buffer (10mM Tris-HCl, pH 8.0, and 0.1 mM EDTA, pH 8.0).

Genomic DNA from progenies was extracted using a 96 well-plate method in which 96 samples were processed simultaneously. Tissue was collected into a 96-well Flat-bottom microtiter plate (Rainin Instrument Co., R96-0APF-1CO) using a standard hole punch. Tissue was kept moist by the addition of 7.5 µl ddH₂O to each well and the plate was kept on ice. A volume of 100 µl of 0.25 N NaOH was added to each well and the tissue was ground for 5 min using a 96-pronged seed crusher. After grinding the tissue, 7.5 µl of ground sample was transferred to a second 96-well plate containing 75 µl of neutralization solution (0.05 M Tris-HCL pH 7.0, 0.1 mM EDTA) for each well of the plate. The samples were covered and stored in the -80°C freezer to prevent degradation. These DNA isolations were stable for one to two weeks.

**Polymerase Chain Reaction assay for genotyping.** Amplification reactions were performed in 20 µl final volume with 3 µl of the suspended DNA template (5 ng per reaction), 12.6 µl of H₂O, 2 µl of PCR reaction buffer (10 mM Tris-HCl, 50 mM KCl), 0.4 µl of MgCl₂ (25 mM), 0.8 µl ddNTPs (0.2 mM of each dNTP), 1.2 µl of MgCl₂ (25 mM), 0.8 µl ddNTPs (0.2 mM of each dNTP), 0.6 µl of primers (50 pmol of each oligonucleotide), and 0.2 µl of Taq DNA polymerase (1 unit per reaction). Reactions were carried out in an automated thermocycler (MJ Research, Inc., Watertown, MA). The
initial step was at 94 °C for 2 min followed by 94 °C for 1 min, the annealing temperature was 55 °C for 1 min and polymerization at 72 °C for 2 min. Cycles varied depending on the primers used (see Table 4.1). Final polymerization was at 72 °C for 5 min. Amplification of PCR and digestion products were visualized by electrophoresis in 2% agarose gels, stained with ethidium bromide, and photographed under UV light.

**Molecular Marker Development.** DNA-based genetic markers that can be detected using the polymerase chain reaction (PCR) were developed using existing cDNA and EST sequences for genes of interest. These sequences were obtained from the National Center for Biotechnology Information (NCBI), and all sequences were downloaded in FASTA format for comparison and primer development. Putative polymorphic regions were confirmed by PCR with restriction enzymes and by size polymorphism. In the case of the dg gene, markers were described by Levin et al. (2002). A marker for the tangerine allele t used sequence information described by Isaacson et al. (2002). A marker for Delta was developed from the sequence of the lycopene ε-cyclase reported by Ronen et al., (1999). Primer design was carried out using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and primers were synthesized and purchased using commercial sources. When databases contained insufficient information to develop markers, alleles of parental lines were sequenced to detect putative single nucleotide polymorphism (SNPs). Restriction enzymes that recognized SNPs were used to detect allelic differences as a Cut Amplified Polymorphisms (CAPs). Deletions and CAPs were visualized by electrophoresis on 2% agarose gel, stained with ethidium bromide as described above. Table 4.1 lists the primers used as markers for this study.
Selection for combination of alleles. Selection for gene combinations was made in F2 generations in the greenhouse using both classical methods and molecular markers. Classical selection techniques included the identification of progenies that were homozygous for the \( t' \) allele based on the light green leaves associated with the virescent phenotype (Figure 4.1) and selection for \( dg \) at the seedling stage under yellow filter (Mochizuki and Kamimura, 1985) (Figures 4.2 and 4.3). Selection based on MAS was applied to crosses involving the high pigment allele \( dg, tangerine, \) and \( Delta \). Selection occurred approximately four weeks after seeding, with MAS used for the identification of progenies homozygous for the \( t \) allele (Figure 4.1A) and the \( Delta \) mutation (Figure 4.1B). Although progenies homozygous for \( dg \) were selected by classical methodologies, they were also tested with molecular markers to confirm genotypes. Selected tomato plants were transplanted into 50-well flats containing metromix with greenhouse and growth conditions as described above and planted in the field at the Horticulture and Crop Science Farm, Wooster, OH.

Carotenoid analysis

Carotenoid extraction. Carotenoid extraction was performed using a modified version of the hexane extraction methods described by Ferruzzi et al., 1998. Briefly, five grams of pureed tomato were weighed on an analytical scale, and 1.0g of calcium carbonate (Precipitated chalk, Fisher) and 4.0g of celite (Celite 545, Fisher) were added. Fifty ml of methanol were added followed by further stirring. The mixture was allowed to stand for approximately 1 min followed by homogenization for 1 min. The methanol
extract was filtered using Whatman paper #1 and # 42 (42 on the bottom), pre wet in methanol. The filtrate was suspended in 50 ml of 1:1 acetone/hexane and allowed to stand for 1 min prior to homogenization. The extract was filtered as before and the filtrate saved. Repeated acetone/hexane extractions (up to three times) were required to recover the majority of lycopene. The liquid phase was pooled, transferred to a separatory funnel and washed with approximately 20 ml of distilled water. The bottom layer (aqueous) was discarded and the rinsing step repeated for the top layer (organic). The top layer (organic) was filtered through sodium sulfate (Sodium sulfate anhydrous, Fisher) and glass wool to remove remaining water, and rinsed with hexane. The final volume was made up to 100 ml with hexane. Five vials with aliquots (3.0 ml) of diluted extract (extract was diluted 1/5 in hexane) were dried under a stream of compress nitrogen to remove the solvent. The vials were wrapped in aluminum foil to protect from light and placed at −20°C until HPLC analysis.

**HPLC separation and carotenoid quantitation.** The chromatographic separations of carotenoids were performed using a VYDAC™ reversed-phase C₁₈ column and an YMC Carotenoid 4.6 x 250 mm C₃₀ column on a Waters 2695 HPLC. The C₃₀ column was used to separate δ-carotene and ζ-carotene for the population that carried both the Delta and t genes. The C₁₈ column was used for all other analysis. Dried extracts were reconstituted with appropriate volumes (3 ml) of MTBE (ether) and methanol (1:2) respectively. Samples of 100 µl were injected to a linear gradient (0 min, 100% of 1% of ammonium acetate in MeOH ; at 20 min, 80% of 1% of ammonium acetate in MeOH and 20% MTBE; at 25 min, 80% of 1% of ammonium acetate in MeOH and 20%
MTBE; at 30 min, 100% of 1% of ammonium acetate in MeOH) with a flow rate of 1 ml/min for 30 min to separate carotenoid pigments. Peaks were detected in a range of 210-700 nm using a Waters 996 photo-diode array detector. Carotenoids were identified by their characteristic absorption spectra in comparison to carotenoid standards. The quantity of each carotenoid was determined by the integration of the peak area using the Empower software (Waters Corporation, 2003).

**Generation of standard curves for tetra-cis lycopene, δ-carotene, and ζ-carotene.** Bulk extraction from 20 g of puree from t x dg selection (FG03-310) was used for isolation of tetra-cis lycopene and ζ-carotene. A similar extraction from selection FG03-307 was used for the isolation of δ-carotene. The ~75 ml hexane extraction (following the carotenoid extraction described above) was concentrated in a rotavapor (Büchi, R110 rotavapor) at 60°C to an approximate volume of 10 ml. The concentrated extract was dried under nitrogen, and the vial containing the dried extract was wrapped in aluminum foil to protect from light prior to storage at -20°C freezer until preparative HPLC separation.

The dried extract was reconstituted in 1 ml of a mixture of MTBE (ether) and methanol (1:2) respectively. Pigments were separated using an hp1050 on a reversed-phase C18 Waters prep column (125Å, 10µm, 19 x 300 mm). One ml of MTBE: MeOH (1:2) concentrated pigment was injected to the hp1050 HPLC pump on the linear MTBE (solvent A) and 1% of ammonium acetate in MeOH (solvent B) gradient at a flow rate of 5 ml/min as follows: at 0 min, 100% solvent B; at 40 min, 80% solvent B and 20% solvent A; at 50 min, 80% solvent B and 20% solvent A; at 60 min, 100% solvent B.
Detection of tetra-\textit{cis} lycopene peak was detected at 438 nm with an elution time of 45 min. Peaks for \(\zeta\)-carotene and \(\delta\)-carotene were detected at 400 nm and 454 nm respectively. Both compounds had an elution time of 51 min. The collected fractions were dried under a stream of compress nitrogen and storage at \(-20^\circ\text{C}\) freezer until preparative for HPLC purity confirmation and standard curve preparation. Purity of isolated pigments was confirmed using the Waters HPLC following the analytical procedure described above.

A stock solution with an absorbance of \(\sim 0.9\) of the pigment of interest was prepared. Serial dilutions were made from the stock solution. After making the serial dilutions, absorbance of each point of the curve was measured in order to generate a standard curve. Concentration was calculated as \((\text{Abs}/\varepsilon)\); where Abs is the absorbance measured and \(\varepsilon\) = molar extinction coefficient of the pigment.

When standard curves were not available as in the cases of phytoene, neurosporene and \textit{cis}-lycopene isomers, concentration was calculated using \(\zeta\)-carotene standard curve for phytoene and neurosporene, and tetra-\textit{cis} lycopene standard curve for \textit{cis}-lycopene isomers. Lycopene and \(\beta\)-carotene standards were purchased from Sigma Chemical Co. (St. Louis, MO).

\textbf{Data analysis.} Quantitative measurements of selected compounds were analyzed using SAS (8.2). All measurements of compounds were standardized to \(\mu\text{g/g}\) on a fresh weight basis and analyzed using the general linear model (GLM) procedure for analysis of variance (ANOVA). The statistical model used for ANOVA was:

\[
Y_{ij} = \mu + \tau_i + \beta_j + \epsilon_{ij}
\]
Where $Y_{ij} =$ concentration of compound of the $j^{th}$ plant grown under the $i^{th}$ level of the factor $\tau$, where $\tau$ is genotype. The term $\mu$ is the population mean, $\beta_j$ is the effect of the $j^{th}$ block and $\epsilon_{ij}$ is the residual error term. Blocks were considered random effects for ANOVA.

Mean comparisons of genotypes were performed using the least significant difference (LSD) procedure. Variance components were estimated using Restricted Maximum Likelihood (REML). The variance of a variable explained by genotype was equal to $V_G / (V_G + V_R + V_E)$. Where $V_G =$ variance due to genotype, $V_R =$ variance due to replication (block), and $V_E =$ variance due to uncontrolled error.

RESULTS AND DISCUSSION

The population sizes per block ranged from 1 to 25 individuals for specific genotypes. For crosses carrying the gene combinations of tangerine ($t$) and dark green ($dg$), a total of 5 plants were selected. For the gene combinations of tangerine virescent ($t'$) and $dg$, 50 plants were selected. Likewise, 50 plants were selected for the gene combinations of Beta and $dg$. Only two plants were selected for the gene combinations of Delta and $dg$. For the combination of Delta with $t$ and $t'$, 4 and 10 plants were selected respectively. Classical selection of $dg$ (seedlings grown under yellow film), and $t''$ (based
on virescent phenotype) proved to be efficient for selecting plants carrying a combination of these genes. Classical selection predicted genotype 99.9% of the time.

DNA-based markers that could distinguish the \( t \) allele of \textit{tangerine}, the \textit{Delta} allele of the lycopene-\( \epsilon \) cyclase, and the \textit{dg} allele of high pigment from wild type alleles were successfully applied to selection and to confirm genotypes. The \( t \) allele from Carolina Gold appears to be identical to the \( t^{3183} \) allele based on the presence of a \( \sim 345 \) bp deletion (Figure 4.1), which corresponds to that described previously (Isaacson et al., 2002). A marker for the \textit{Delta} allele was developed based on a single nucleotide polymorphism that could be recognized by the enzyme \textit{Hind III}. This polymorphism was located in an intron flanked by two exons. The recognized sequence in \textit{Delta} was 5' -AAGCTT-3', while in the wild type the last T was replaced by a C (5'-AAGCTC-3').

Phenotypic evaluation of parents, selected progeny, and genetic stocks was conducted using HPLC to determine the profile of carotenoid pigments with attention focused on concentration and isomeric structure. Chromatographic representation of carotenoids for the different gene combinations are presented in Figure 2.2. Concentration of carotenoid pigments in varieties, lines, progeny selected for combinations of genes and genetic stocks are listed in Table 2.2.

The concentration of trans-lycopene varied from 0 to 132.7 \( \mu g/g \) fresh weight. Variety FG99-218 had the highest concentration of lycopene (\( P < 0.0001 \)) and elevated \( \beta \)-carotene (\( P < 0.0001 \)) relative to wild type controls as a result of the presence of \textit{dg} and
Analysis of variance components showed that variation due to genetic differences explained 99.5% of the total variation in lycopene concentration while environment and differences associated with blocks explained 0.5%.

The concentration of β-carotene varied from 0 to 52.7 µg/g fresh weight. Selection FG03-308, developed by combining the Beta allele with dg, had approximately 25% more β-carotene than the Beta parent, 150% more than the dg and ogc parent, and ten fold more than standard varieties (Table 2.4). ANOVA indicated that these difference in β-carotene concentration were highly significant (P= 0.0011). Genetic differences explained 99.4% of the total variation in β-carotene concentration while environmental differences associated with blocks was not significant (P= 0.3813) explaining only 0.09% of the total variation. These results demonstrated that when Beta is combined with dg the concentration of β-carotene in tomato fruits is augmented.

Levels of δ-carotene varied between 0 and 45.8 µg/g fresh weight. Selection FG03-307, which combined Delta with dg had a 5% increase in δ-carotene relative to the Delta parent, and this difference was significant ((P= 0.0034) based on direct contrasts (data not shown). Genotype explained 94.5% of the total variation in δ-carotene concentration while block explained 0.001%, and uncontrolled error 5.5%. The combination of Delta with dg also resulted in an incremental increase of this carotenoid. These findings support the role of dg in increasing total carotenoid levels (Levin et al., 2003).

Tetra-cis lycopene concentration varied from 0 to 52.8 µg/g fresh weight. Selections FG03-310 and FG03-311, which combined the l' allele with dg, were not
significantly different in tetra-cis lycopene concentration relative to their \( t' \) parents. However, these selections have more carotenoid precursors than other selections. FG03-310 and FG03-311 have approximately 2 fold more phytoene, 3 fold more \( \zeta \)-carotene, and 2 fold more neurosporene than their \( t' \) parents (Table 2.3). These increases in precursors were significant (P= 0.0060, 0.0097, and <0.0001 respectively). Selection FG03-301, which combined the \( t \) allele with \( dg \), has a significantly higher concentration of tetra-cis lycopene and carotenoid precursors (phytoene, \( \zeta \)-carotene, and neurosporene) relative to its \( t \) parent (Table 2.3). The variances attributed to the genetic differences were 86.4%, 80.5%, 83.2%, 95% for tetra-cis lycopene, \( \zeta \)-carotene, phytoene, and neurosporene respectively. No variance was attributed to blocking criteria, and the rest of the variance was attributed to uncontrolled error. These findings suggest that when genes affecting chromoplast quantity (\( dg \)) are combined with the \( t' \) allele, carotenoid concentration is shifted toward precursors instead of higher amount of tetra-cis lycopene. The reason for the augmentation of carotenoid precursors at the expense of higher concentration of tetra-cis lycopene when the \( t' \) allele is present is unclear. When \( dg \) is combined with the \( t \) allele, the total carotenoid concentration is elevated as we predicted based on previous reports (Levin et al., 2003). Isaacson et al (2002) hypothesized that the enzyme that converts tetra-cis lycopene to all-trans lycopene, carotenoid isomerase (CRTISO), works together with \( \zeta \)-carotene deasturase (ZCD) forming a complex. This hypothesis could explain the accumulation of carotenoid precursors suggesting that in the absence of a functional CRTISO, ZCD does not efficiently transform \( \zeta \)-carotene to tetra-
cis lycopene leading to the accumulation of carotenoid precursors. This effect appears to be more severe for the \( t' \) allele than the \( t \) allele.

Combination of the gene \( \text{Delta} \) with genes affecting carotenoid isomerization (\( t \) and \( t' \)) suggest that lycopene \( \varepsilon \)-cyclase does not efficiently use tetra-\( cis \) lycopene as a substrate and convert it to \( \delta \)-carotene (Table 2.6). Selections that combine \textit{tangerine} alleles with \( \text{Delta} \) (FG03-401 and FG03-404) resulted in 77-88\% decrease in \( \delta \)-carotene as compared to the \( \text{Delta} \) parent (\( P = 0.0002 \)). Selection FG03-401 (\( \text{Delta} \times t \)) resulted in a significantly higher amount of tetra-\( cis \) lycopene (\( P = 0.0001 \)) relative to the \( t \) parent but no significant difference in the concentration of \( \zeta \)-carotene. Selection FG03-404 (\( \text{Delta} \times t' \)) did not show a significant difference in tetra-\( cis \) lycopene concentration, however the concentration of \( \zeta \)-carotene in this line was significantly lower (\( P = 0.0067 \)) when compared to its \( t' \) parents (Table 2.6). These results show that lycopene in the \( cis \) configuration cannot be cyclized as efficiently as the \textit{trans}-lycopene and support the hypothesis that the lycopene cyclases cannot utilize tetra-\( cis \) lycopene as a substrate because of steric hindrance (Isaacson et al., 2002). This inefficiency in the cyclization reaction with \( cis \) substrate lead to a decreased in the production of \( \delta \)-carotene even when the enzyme mediating this reaction was over expressed.

The developed tomato genetic resource with a wide diversity in carotenoid structure and concentration may allow the testing of specific physiological hypotheses. Lines ranged in carotenoid concentration from 0-132 \( \mu g/gm \) lycopene, 0-52 \( \mu g/gm \) \( \beta \)-carotene, 0-45 \( \mu g/gm \) \( \delta \)-carotene, 0-52 \( \mu g/gm \) tetra-\( cis \) lycopene, 0-62 \( \mu g/gm \) \( \zeta \)-carotene, 0-45 \( \mu g/gm \) phytoene, and 0-15 \( \mu g/gm \) neurosporene on a fresh weight basis. This
germplasm will provide the raw material for further use in research to address questions concerning the bioavailability of phytonutrients using in vitro and in vivo models. For example, previous research demonstrated that both lycopene and carotenoid precursors represent a potent compounds for anti-cancer research (Nara et al., 2001). This research demonstrated that \( \zeta \)-carotene and phytofluene were highly efficient at inhibiting the growth of HL-60 human promyelocytic leukemia cells by inducing apoptosis with \( \zeta \)-carotene being the most effective. The effect of these compounds on the cell growth inhibition was concentration dependant with higher concentrations being more effective.

Other carotenoids are of dietary interest due to their role as nutrients. Vitamin A deficiency remains a major cause of blindness, in developing countries preschool children and women of reproductive age being the most affected (Hedrén et al., 2002).

One implication of the genetic resources described and characterized in this research is that even simple genetic changes may affect multiple carotenoids and carotenoid precursors. These results further accentuate the significance of genetic interactions on carotenoid concentration in tomato fruits. In order to assess the physiological significance of bioactive components in the food matrix, it will be necessary to blend product from multiple lines to control concentrations and ratios of the carotenoids under study. For example, test diets for the effects of \( \zeta \)-carotene, phytoene, and neurosporene independently from tetra-cis-lycopene could be developed from line 02-1023-1 (low in \( \zeta \)-carotene, phytoene, and neurosporene) by the addition of a small amount of no carotenoid product (line 03-6336) to reduce the concentration of cis-lycopene and comparing that mixture to product from line FG03-301 (high in \( \zeta \)-carotene,
phytoene, and neurosporene). Alternatively, the effects of high or low cis-lycopene diets could be studied in lines 02-1025-1 and Carolina Gold after correcting for precursors by blending in no carotenoid product. The information gained from physiological research may then help produce the functional crops needed for the coming generations.
Figure 2.1. Marker assisted selection for \( t \) and \( \text{Delta} \). (A) Selection of \( t \) based on the presence of a \( \sim345 \) bp deletion in the promoter, which corresponds to that previously described (Isaacson et al., 2002). \textit{Tangerine} (\( t \)) codes for a non functional carotenoid isomerase (CRTISO) and is an allele of \textit{tangerine virescent} (\( t^v \)). The presence of \( t \) in tomato leads to the accumulation of tetra-\textit{cis} lycopene at the expense of \textit{trans}-lycopene. (B) Selection of \textit{Delta} based on a single nucleotide polymorphism (SNP) that could be recognized by the enzyme \textit{Hind} III. \textit{Delta} codes for a lycopene \( \varepsilon \)-cyclase, the presence of \textit{Delta} in tomato enhances the concentration of \( \delta \)-carotene at the expense of \textit{trans}-lycopene.
Figure 2.2. Classical methods for genotype selection. (A) Selection of \( t^r \) based on the yellowing of young leaves characteristic of the virescent phenotype. The allele \( t^r \), \textit{tangerine virescent}, codes for a non-functional carotenoid isomerase (CRTISO) and is an allele of \textit{tangerine} (\( t \)). The presence of \( t^r \) and \( t \) in the homozygous condition leads to the accumulation of tetra-cis lycopene at the expense of trans-lycopene. (B) Alternative selection for the \( dg \) gene using a photo-selective method as described by Mochizuki and Kamimura, 1985. The \textit{dark green} (\( dg \)) codes for the tomato homolog of the DEETIOLATED 1 (DET 1) and is an allele of \textit{high pigment-2} (\( hp-2 \)). The presence of \( dg \) in tomato increases the number of chromoplasts as well as the total carotenoid concentration of fruit. (C) Presence of anthocyanins and lack of spindly (etiolated) growth under yellow film selection indicate homozygous \( dg \) plants.
Table 2.1. List of primers used as markers to detect the presence of genes affecting carotenoid concentration and structure. \(^1\)Dark green (dg) codes for a defective version of the tomato homolog of DEETIOLATED 1 and is an allele of high pigment-2 (hp-2), the presence of dg in tomato increases the number of chromoplasts; Delta codes for a lycopene \(\epsilon\)-cyclase, the presence of Delta in tomato enhances the content of \(\delta\)-carotene at the expense of trans-lycopene; tangerine codes for a non-functional carotenoid isomerase (CRTISO), this mutation in tomato leads to the accumulation of tetra-cis lycopene at the expense of trans-lycopene. \(^2\)CAP, cut amplified polymorphism; Size pm, size polymorphism.

<table>
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<th>Marker type (^2)</th>
<th>Temp ((^°)C)</th>
<th>Cycles</th>
<th>Enzyme</th>
</tr>
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<td>55</td>
<td>36</td>
<td>Acl</td>
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<tr>
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<td>5'-CACCAATGCTATGTCGCAA-3'</td>
<td>dg</td>
<td>CAP</td>
<td>55</td>
<td>36</td>
<td>Acl</td>
</tr>
<tr>
<td>DlpF1</td>
<td>5'-TGGCAAGGTGATGTTGAT-3'</td>
<td>Delta</td>
<td>CAP</td>
<td>55</td>
<td>38</td>
<td>Hind III</td>
</tr>
<tr>
<td>DlpR1</td>
<td>5'-CTCCTGTCGAATGGACCTGT-3'</td>
<td>Delta</td>
<td>CAP</td>
<td>55</td>
<td>38</td>
<td>Hind III</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>44</td>
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Table 2.2. Carotenoids detected in the fruits of different tomato lines and varieties including lines developed for this study.  
\(^1\)Lines are indicated by Ohio accession numbers (FG-, F6-, G1-, 02-, and 96-); Tomato Genetics Resource Center Lycopersicon accession number (LA); USDA designation (97L97), or variety name (Carolina Gold, North Carolina State University). \(^2t\), \textit{tangerine} codes for a non functional carotenoid isomerase (CRTISO) and is an allele of \textit{tangerine virescent (t)}\(^\prime\), the presence of \textit{t} and \textit{t}\(^\prime\) in tomato leads to the accumulation of tetra-cis lycopene instead of \textit{trans}-lycopene. \textit{Dark green (dg)} codes for a defective homolog of the DEETIOLATED 1 gene and is an allele of \textit{high pigment-2 (hp-2)}. The presence of \textit{dg} in tomato increases the number of chromoplasts and the total carotenoid levels. \textit{Beta} codes for a lycopene \(\beta\)-cyclase and is an allele of \textit{old gold crimsom (og)}\(^5\), the presence of \textit{Beta} in tomato leads to the accumulation of \(\beta\)-carotene at the expense of \textit{trans}-lycopene while \textit{ogc} increases lycopene content. \textit{Delta} codes for a lycopene \(\epsilon\)-cyclase, the presence of this gene in tomato leads to the accumulation of \(\delta\)-carotene at the expense of \textit{trans}-lycopene. The gene \textit{r} encodes a defective phytoene synthase and \textit{wt} indicates the \textit{wild type} or normal allele. \(^3\)Quantification was based on \(\zeta\)-carotene standard curve. \(^4\)Quantification was based on tetra-cis lycopene standard curve. 
Values entered as "0" were below the level of detection.
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<td>t°</td>
</tr>
<tr>
<td>02-1025-1</td>
<td>t°</td>
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<tr>
<td>Gold'</td>
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</tr>
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<td>wt</td>
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<td>96-2472</td>
<td>wt</td>
</tr>
<tr>
<td>FG99-218</td>
<td>wt</td>
</tr>
<tr>
<td>FG03-310</td>
<td>t°</td>
</tr>
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<td>t°</td>
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</tr>
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Figure 2.3. HPLC separation of the carotenoids present in fruit of selections from crosses to combine genes affecting carotenoid structure and concentration (t, Beta, and Delta) and genes affecting chromoplast quantity (dg). Tangerine (t) and tangerine virescent (t') are alleles that code for non functional carotenoid isomerase (CRTISO). The presence of these alleles in tomato leads to the accumulation of tetra-cis lycopene instead of trans-lycopene. Beta codes for a lycopene β-cyclase and is an allele of old gold crimsom (og^5). The presence of Beta leads to the accumulation of β-carotene at the expense of trans-lycopene and og^5 has the contrary effect. Delta codes for a lycopene ε-cyclase that leads to the accumulation of δ-carotene at the expense of trans-lycopene. Dark green (dg) codes for a non-functional homolog of the DEETIOLATED gene and is an allele of high pigment-2 (hp-2). The presence of dg in tomato increases the number of chromoplasts and increases carotenoid levels. Wild type, wt, indicates the standard allele found in red tomatoes. Peak identification: tetra-cis lycopene (1), phytoene (2), ζ-carotene (3), neurosporene (4), β-carotene (5), δ-carotene (6), and lycopene (7).
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<td>t</td>
</tr>
<tr>
<td>FG99-218*</td>
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<td>Carolina gold*</td>
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</tr>
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<td>t</td>
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<tr>
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</tr>
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Table 2.3. Comparison of carotenoid concentration of selections FG03-310, FG03-311 and FG03-301, and parents*1.1 Lines are indicated by Ohio accession numbers (FG and 02), and variety name (Carolina Gold, North Carolina State University).

2t, tangerine codes for a non-functional carotenoid isomerase (CRTISO) and is an allele of tangerine virescent (t'), the presence of this gene in tomato leads to the accumulation of tetra-cis lycopene instead of trans-lycopene; dg, dark green codes for a non-functional tomato homolog of the DEETIOLATED 1 (DET 1) gene and is an allele of high pigment-2 (hp-2), the presence of dg in tomato increases the number of chromoplast and the total carotenoid levels; og*, old gold crimson codes for a lycopene β-cyclase and is an alleles of Beta, the presence of this mutation in tomato leads to the accumulation of trans-lycopene at the expense of β-carotene, wt, wild type. 3Quantification was based on ζ-carotene standard curve. 4Quantification was based on tetra-cis lycopene standard curve. Values entered as "0" were below the level of detection.
Table 2.4. Comparison of lycopene and β-carotene concentration of selection FG03-308 and parents*. 1 Lines are indicated by Ohio accession numbers (FG) and USDA designation (97L97). 2 Beta codes for a lycopene β-cyclase and is an alleles of old gold crimson (ogc), the presence of this mutation in tomato leads to the accumulation of β-carotene at the expense of trans-lycopene; dg, dark green codes for a non-functional homolog of the DEETIOLATED 1 (DET 1) gene and is an allele of high pigment-2 (hp-2), the presence of dg in tomato increases the number of chromoplast along with total carotenoid levels; wt, wild type.
Values entered as “0” were below the level of detection.

<table>
<thead>
<tr>
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<th>µg/g fresh weight</th>
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<tbody>
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<td>FG03-308</td>
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<td>P-value</td>
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</table>

Table 2.5. Comparison of carotenoid concentration of selection FG03-307 and parents*. 2 Delta codes for a lycopene ε-cyclase, the presence of this mutation in tomato leads to the accumulation of δ-carotene at the expense of trans-lycopene; dg, dark green codes for a non-functional homolog of the DEETIOLATED 1 (DET 1) gene and is an allele of high pigment-2 (hp-2), the presence of dg in tomato increases the number of chromoplast as well as the total carotenoid levels; ogc, old gold crimson codes for a lycopene β-cyclase and is an alleles of Beta, the presence of this mutation in tomato leads to the accumulation of trans-lycopene at the expense of β-carotene; wt, wild type.
Values entered as “0” were below level of detection.

<table>
<thead>
<tr>
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<tbody>
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57
Table 2.6. Comparison of carotenoid concentration of selections FG03-401 and FG03-404 with respect to their parents*, in response to tetra-cis lycopene cyclization during steric hindrance.  

1 Lines are indicated by Ohio accession numbers (FG and 02); USDA designation (97L97), or variety name (Carolina Gold, North Carolina State University).  

2 t, tangerine codes for a non functional carotenoid isomerase (CRTISO) and is an allele of tangerine virescent (t'), the presence of this gene in tomato leads to the accumulation of tetra-cis lycopene; Delta codes for a lycopene £-cyclase, the presence of this mutation in tomato leads to the accumulation of 8-carotene at the expense of trans-lycopene; age, old gold crimpson codes for a lycopene P­ cyclase and is an allele of Beta, the presence of this mutation in tomato leads to the accumulation of trans-lycopene at the expense of P-carotene.  

3 Quantification was based on 8-carotene standard curve.  

4 Quantification was based on tetra-cis lycopene standard curve.  

Values entered as “O” were below the level of detection.

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<th>phytoene</th>
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<td>wt</td>
<td>ogc</td>
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<td>wt</td>
<td>ogc</td>
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LSD 0.05  

| P-value | 2.8 | 9.3 | 10.1 | 5.7 | 4.8 | 6.4 | 5.8 | 0.0001 | 0.0002 | 0.0001 | 0.0007 | 0.0013 | 0.0142 | 0.0109 |

Values entered as “0” were below the level of detection.
Figure 2.4. Conversion of tetra-cis lycopene to δ-carotene is inefficient relative to the cyclization of trans-lycopene. *Delta* codes for a lycopene ε-cyclase, the presence of this allele in tomato leads to the accumulation of δ-carotene at the expense of trans-lycopene. *Tangerine* (*t*) and *tangerine virescent* (*t'*) are alleles that code for a non functional carotenoid isomerase (CRTISO) and lead to the accumulation of tetra-cis lycopene. Peak identification: tetra-cis lycopene (1), and δ-carotene (2).
REFERENCES


CHAPTER 3

Effect of genetic background on carotenoid profiles in tomato

(Lycopersicon esculentum Mill.) fruits

ABSTRACT

Tomato serves as a model organism for investigating the role of plant pigments in human nutrition. Several well documented genetic variants exist that allow the manipulation of carotenoid content, structure, and concentration using traditional crossing and selection employed by plant breeders. However it is not well known how other genes that plant breeders directly manipulate and uncharacterized genes associated with genetic background affect carotenoid concentration, structure, and content and thus the nutritional quality of tomato fruit. As part of an effort to understand how the genetic background may affect the carotenoid composition in tomato fruits, lines carrying rin and the tangerine (t) allele were crossed to lines carrying the tangerine virescent (t') allele. DNA-based molecular markers designed to distinguish alleles of rin and t were used to screen the segregating population and progenies were characterized for carotenoid composition using HPLC. The presence of the rin mutation had a significant effect on carotenoid profile. When rin was present in the homozygous condition, the predominant
lycopene isomer in tangerine tomatoes, tetra-cis lycopene, decreased by 91%. There was a 45% decrease in tetra-cis lycopene when rin was in the heterozygous condition, demonstrating that hybrids employing the rin locus to impart long shelf life may have a lower level of certain carotenoids. An increase in β-carotene was observed when the rin mutation was present in both the homozygous and heterozygous condition in the tangerine background, with the homozygous rin lines having significantly more β-carotene than the heterozygous. No ζ-carotene and neurosporene were detected when rin was present in the homozygous condition, and these compounds decreased 42% and 49% respectively, relative to wild type plants when rin was in the heterozygous condition.

When rin was in the homozygous and heterozygous condition, phytoene decreased 88% and 51% respectively, relative to wild type plants. The allele present at the tangerine locus did not have a significant effect on the profile of carotenoid precursors, but the concentration of tetra-cis lycopene and β-carotene did vary, with lines carrying the t allele containing more β-carotene and lines carrying the t' allele containing more tetra-cis lycopene.

INTRODUCTION

Variation in the concentration and isomeric structure of specific carotenoids exists within varieties of tomato. The genetic control of pigment concentration and structure is influenced by structural genes that mediate the biochemical synthesis of carotenoids, by the regulation of these genes, and by genes that affect the structure and density of the chromoplast. Genes that affect the biochemical synthesis of carotenoids include the
tangerine alleles (t and t'), alleles of the lycopene β-cyclase (Beta and og), and an allele of the lycopene ε-cyclase (Delta). The tangerine mutation is caused by the production of a non-functional carotenoid isomerase (CRTISO) leading to the accumulation of tetra-cis lycopene at the expense of trans-lycopene. This enzyme converts tetra-cis lycopene into trans-lycopene, and is thought to work in a complex with ζ-carotene desaturase (ZCD) (Isaacson et al., 2002). The Beta mutation leads to the accumulation of β-carotene at the expense of lycopene. This hyper accumulation of β-carotene is caused by an over expression of the enzyme mediating the β- cyclization reaction, lycopene β-cyclase. The old gold crimpson (og) allele has the opposite effect to that of Beta, with lycopene accumulating due to the lack of β- cyclase activity. Another gene that affects carotenoid biosynthesis is the Delta mutation which leads to the accumulation of δ-carotene at the expense of lycopene. This mutation is caused by an over expression of the lycopene ε-cyclase.

Genes that affect the biochemical synthesis of carotenoids are of interest in developing genomic resources to test physiological effects of carotenoids in the human diet. For example, lycopene mainly exists in the trans configuration while in the human plasma lycopene exists as an isomeric mixture after consumption of red tomato or tomato products (van Breemen et al., 2002; Stahl and Sies, 1996). It has been reported that when lycopene is ingested in the trans form, it is poorly absorbed while its cis isomer is more readily absorbed (Unlu et al., 2003). Tomato fruit with pigments in a cis configuration are encountered in the fruit of varieties carrying the tangerine gene but there is a lack of
knowledge concerning how the structure of lycopene affects its bioactivity and uptake in the human body.

Genes that affect structure and density of chromoplasts include the \textit{green flesh} (gf), and the \textit{high pigment} genes, including the alleles of \textit{high pigment-2, dark green} (dg). The mutation caused by the \textit{gf} gene alters the chloroplast-chromoplast transition leading to the retention of chlorophyll as the plastid accumulates carotenoids. There are data in the literature that suggest \textit{gf} tomatoes also have higher lycopene content (Akhtar et al., 1999). The mutation caused by \textit{dg} increases the chloroplast level in green tissues and the chromoplast density in fruit, leading to the augmentation of total carotenoid levels. Thus carotenoid content can be varied by genes that do not directly affect the biosynthetic pathway.

Among other processes that affect the accumulation of carotenoids in fruit, ripening plays an essential role. There has been an extensive use of genes that modify the ripening process by plant breeders in order to develop long-shelf-life (LSL) tomato varieties. Mutants affecting ripening include: \textit{ripening inhibitor} (\textit{rin}, on chromosome 5), \textit{non-ripening} (\textit{nor}) and \textit{alcobaca} (\textit{alc}) on chromosome 10, and \textit{never ripe} (\textit{Nr}, on chromosome 9) (Tigchelaar et al., 1978; McGlasson, 1985). These non-ripening mutants affect the process of ripening in different ways. Although all four genes slow the ripening process, varying amount of physiological and molecular information is available concerning their action (Robinson and Tomes, 1968; Tigchelaar et al., 1978; Vrebalov et al., 2002; Lincoln and Fischer, 1988; Murray et al., 1995; Atta-Aly et al., 1998; Atherton and Rudich, 1986). The genes \textit{rin} and \textit{Nr} have been isolated, and their DNA sequence
determined. *Nr* codes for a mutation in the ethylene receptor that is active in fruit. The *rin* mutation occurred spontaneously in a breeding line developed at Cornell University by H. Munger (Robinson and Tomes, 1968). This mutation leads to the inhibition of all ripening phenomena and is caused by a deletion that results in the production of a chimeric RNA for a regulatory protein, *LeMADS-RIN*. The mutation resulting in the inhibition or normal *Rin* functions such that climacteric respiration cannot be initiated and ripening cannot be completed (Vrebalov et al., 2002). In addition, plants with the *rin* mutation lack determinate inflorescence and have enlarged sepals, (Lincoln and Fischer, 1988; Vrebalov et al., 2002). Hybrid varieties of tomato that use *rin* and *alcobaca* are in commercial production due to the increased field and shelf storage, and shipping capacity of fruit that are heterozygous for these genes (Nguyễn et al., 1991).

Despite the extensive use of *rin* in breeding it has not been reported how this allele affects the carotenoid profile in tomato fruits. The aim of the present work was to assess the effect of the genetic background on carotenoid concentration in tomato fruits in order to determine how genetic changes targeted by plant breeders may affect the nutritional and functional food potential of varieties.

**MATERIALS AND METHODS**

*Germlasm and population design.* Populations were developed by crossing inbred lines Ohio 02-1023 and Ohio 02-1025 to NC99471-4 (R. Gardner, North Carolina State University). Both Ohio lines contain the *t"* allele introduced from the genetic stock LA0351 into Ohio 9242. The line NC99471-4 contains the *t* allele at the *tangerine* locus
and the *rin* gene. Hybrids from these crosses, FG02-212 for crosses Ohio 02-1025 and FG01-214 with Ohio 02-1023, were self pollinated to produce segregating F$_2$ populations.

**Experimental design.** The experiments performed for this study included two phases, a genotyping phase, and a testing phase in which carotenoid structure, and concentration were measured. Genotyping occurred in segregating F$_2$ populations after they were transplanted to the field. Progeny were grown in a completely randomized design where genotype was the unit of replication. Parent plants and experimental hybrids were grown in randomized complete block design with plants grown at two locations with two blocks per location.

**Greenhouse and field growth conditions.** Segregating populations (F$_2$) were seeded into 288-well flats containing Metro Mix 360 soil media (Premier Horticulture Inc., Red Hill, PA). Plants were grown in a greenhouse with a day temperature of 23.8° ±3°C and a night temperature of 18.3° ±3°C with a 14/10 h light/dark period. Supplemental high-intensity discharge lighting was provided when natural light fell below 200 W/m$^2$. Seedlings were fertilized twice per day with 20 ppm N-P-K (Peters Professional All-Purpose Fertilizer, 20-20-20, Scotts-Sierra Horticultural Products Co., Marysville, OH). One hundred F$_2$ progeny from the Ohio 02-1025 cross (population A) and 104 from the Ohio 02-1023 cross (population B) were planted in the field at the Horticulture and Crop Science Farm, Wooster, OH following the procedures described in the Ohio Vegetable Production Guide (Precheur, 2000). Progeny were planted with 60 cM spacing between plants within rows, and 1.47 meter spacing between rows. Parents,
and F₁ hybrids used as controls were planted in plots consisting of twenty plants with 30 cM spacing between plants and 1.47 meter spacing between rows. These plots were replicated using a randomized complete block design.

**Genomic DNA extraction for genetic analysis.** Genomic DNA was extracted from young single leaflets collected from plants grown in the field. Each leaflet was ground in a polyethylene sample bag (0.004 gauge) with 1 ml of extraction buffer (0.35 M Sorbitol, 0.1 M Tris, 0.005 M EDTA, pH 7.5, 3.8 g/l NaBisulfite added just before use) by gentle rubbing. 300 µl of the extraction were transferred to a 1.5 ml tube, 300 µl of lysis buffer (0.2 M Tris, 0.05 M EDTA, 2.0 M NaCl, 2% CTAB, pH 7.5) and 150 µl of 5% sarcosine were added. Samples were then incubated at 65 °C for 15 min. After incubation, 700 µl of chloroform: isoamyl alcohol (24:1) were added and tubes were centrifuged for 15 min at 13800 g. The aqueous phase was removed to a fresh 1.5 ml tube and the chloroform:isoamyl alcohol extraction was repeated one more time. DNA was precipitated from the aqueous phase by the addition of 0.8 volume of isopropanol followed by centrifugation at 13800 g for 15- 20 min. After centrifugation, precipitated DNA was dried and resuspended in 500 µl of T1/10 E buffer (10mM Tris-HCl, pH 8.0, and 0.1 mM EDTA, pH 8.0).

**Polymerase Chain Reaction assay for genotyping.** Amplification reactions were performed in 20 µl final volume with 2 µl of the DNA template (5 ng per reaction), 12.6 µl of H2O, 2 µl of PCR reaction buffer (10 mM Tris-HCl, 50 mM KCl), 0.4 µl of MgCl₂ (25 mM), 0.8 µl dNTPs (0.2 mM of each dNTP), 0.6 µl of primers (50 pmol of each oligonucleotide), 1 µl Dimethyl sulfoxide (DMSO) and 0.2 µl of Taq DNA polymerase (1
unit per reaction). Reactions were carried out in an automated thermocycler (MJ Research, Inc., Watertown, MA). Primers and PCR amplification conditions are described in Table 3.1. Amplifications of PCR product were visualized by electrophoresis in 2% agarose gels and stained with ethidium bromide, and photographed under UV light.

**Carotenoid analysis**

*Extraction of carotenoid pigments.* Carotenoid extraction was performed using a modified version of the hexane extraction methods described by Ferruzzi et al., 1998. Briefly, five grams of pureed tomato were weighed on an analytical scale, and 1.0g of calcium carbonate (Precipitated chalk, Fisher) and 4.0g of celite (Celite 545, Fisher) were added. Fifty ml of methanol were added followed by further stirring. The mixture was allowed to stand for approximately 1 min followed by homogenization for 1 min. The methanol extract was filtered using Whatman paper #1 and # 42 (42 on the bottom), pre wet in methanol. The filtrate was suspended in 50 ml of 1:1 acetone/hexane and allowed to stand for 1 min prior to homogenization. The extract was filtered as before and the filtrate saved. Repeated acetone/hexane extractions (up to three times) were required to recover the majority of lycopene. The liquid phase was pooled, transferred to a separatory funnel and washed with approximately 20 ml of distilled water. The bottom layer (aqueous) was discarded and the rinsing step repeated for the top layer (organic). The top layer (organic) was filtered through sodium sulfate (Sodium sulfate anhydrous, Fisher) and glass wool to remove remaining water, and rinsed with hexane. The final
volume was made up to 100 ml with hexane. Five vials with aliquots (3.0 ml) of diluted extract (extract was diluted 1/5 in hexane) were dried under a stream of compressed nitrogen to remove the solvent. The vials were wrapped in aluminum foil to protect from light and placed at -20°C until HPLC analysis.

**HPLC separation and carotenoid quantification.** The chromatographic separations of carotenoids were performed using a VYDAC™ reversed-phase C<sub>18</sub> column on a Waters 2695 HPLC. Dried extracts were reconstituted with appropriate volumes (3 ml) of MTBE (ether) and methanol (1:2) respectively. Samples of 100 µl were injected to a linear gradient (0 min, 100% of 1% of ammonium acetate in MeOH; at 20 min, 80% of 1% of ammonium acetate in MeOH and 20% MTBE; at 25 min, 80% of 1% of ammonium acetate in MeOH and 20% MTBE; at 30 min, 100% of 1% of ammonium acetate in MeOH) with a flow rate of 1 ml/min for 30 min to separate carotenoid pigments. Peaks were detected in a range of 210-700 nm using a Waters 996 photo-diode array detector. Carotenoids were identified by their characteristic absorption spectra in comparison to carotenoid standards. The quantity of each carotenoid was determined by the integration of the peak area using the Empower software (Waters Corporation, 2003).

**Generation of standard curves for tetra-cis lycopene and ζ-carotene.** Bulk extraction from 20 g of puree from t' x dg selection (FG03-310) was used for isolation of tetra-cis lycopene and ζ-carotene. Briefly, the ~75 ml hexane extraction (following the carotenoid extraction described above) was concentrated in a rotavapor (Büchi, R110 rotavapor) to an approximate volume of 10 ml. The concentrated extract was dried under
nitrogen, and the vial containing the dried extract was wrapped in aluminum foil to protect from light prior to storage at -20°C freezer until preparative HPLC separation.

The dried extract was reconstituted in 1 ml of a mixture of MTBE (ether) and methanol (1:2) respectively. Pigments were separated using an hp1050 on a reversed-phase C_{18} Waters prep column (125Å, 10µm, 19 x 300 mm). One ml of MTBE: MeOH (1:2) concentrated pigment was injected to the hp1050 HPLC pump on the linear MTBE (solvent A) and 1% of ammonium acetate in MeOH (solvent B) gradient at a flow rate of 5 ml/min as follows: at 0 min, 100% solvent B; at 40 min, 80% solvent B and 20% solvent A; at 50 min, 80% solvent B and 20% solvent A; at 60 min, 100% solvent B. Detection of tetra-cis lycopene peak was at 438 nm and elution time of 45 min. Peak for ζ-carotene was detected at 400 nm and collected at 51 min. The collected fractions were dried under a stream of compress nitrogen and storage at -20°C freezer until preparative for HPLC purity confirmation and standard curve preparation. Purity of isolated pigments was confirmed using the Waters HPLC following the analytical procedure described above.

A stock solution with an absorbance of ~ 0.9 of the pigment of interest was prepared. Serial dilutions were made from the stock solution. After making the serial dilutions, absorbance of each point of the curve was measured in order to generate a standard curve. Concentration = (Abs/ε); where Abs is the absorbance measured and ε= molar extinction coefficient of the pigment. When standard curves were not available as in the cases of phytoene, neurosporene and lycopene-cis isomers, concentration was calculated using ζ-carotene standard curve (phytoene and neurosporene) and tetra-cis
lycopene standard curve (lycopene-\(cis\) isomers). Lycopene and \(\beta\)-carotene standards were purchased from Sigma Chemical Co. (St. Louis, MO).

**Data analysis.** The standardization of the measurements for all compounds was: \(\mu g/g\) on a fresh weight basis. The collected data was analyzed using the SAS (version 8.2; SAS institute, Cary, NC) general linear model (GLM) procedure for analysis of variance (ANOVA). The statistical model used for parents analysis was:

\[ Y_i = \mu + \tau_i + \varepsilon_i \]

Where \(Y_i\) = concentration of compound of a plant grown under the \(i^{th}\) level of the factor, \(\mu\) is the population mean, \(\tau\) is genotype, and \(\varepsilon_i\) is the residual error term. Using this model, the variance of a variable explained by genotype (\(rin\) or \(t\)) was equal to the \(r^2\), such that \(r^2\) provides an estimate for the proportion of genetic variance explained.

Variance explained by genotype was equal to \(V_G / (V_G + V_E)\) where \(V_G\) = variance due to genotype, and \(V_E\) = variance due to environment. Mean comparison of genotypes was performed using the last significant difference (LSD) procedure.

For progenies, the same model was used was:

\[ Y_i = \mu + M_i + \varepsilon_i \]

Where, \(Y_i\) = concentration of compound of a plant grown under the \(i^{th}\) level of the factor \(M\), \(\mu\) is the population mean, \(M\) is marker, and \(\varepsilon_i\) is the residual error term.
RESULTS AND DISCUSSION

Concentration of carotenoid pigments in the inbred lines and hybrids used to develop the segregating populations under study are listed in Table 4.2. Pigment concentration varies from 1.2-52.8 µg/g tetra-cis lycopene, 1.7-14.6 µg/g ζ-carotene, 0-9.6 µg/g β-carotene, 0.9-4.8 µg/g neurosporene, and 1.0-15.5 µg/g phytoene on a fresh weight basis. Lines carrying the \( t^r \) allele at the tangerine locus and carrying the \( wt \) allele for rin locus had the highest concentration of pigments but the lowest concentration of β-carotene while lines carrying the combination of the \( t \) and rin alleles had the highest concentration of β-carotene and the lowest concentration of other carotenoid pigments. When both genes were present in the heterozygous condition in hybrids, a 54% decrease (P <0.0001) in tetra-cis lycopene relative to lines carrying the \( t^r \) allele occurred. No significant differences were encountered in the other pigments when both alleles were in the heterozygous condition with respect to \( wt \) lines for rin. Similar effects for the rin mutation were reported by Minoggio et al., 2003 where they evaluated the polyphenol pattern of different tomato lines. They found that when rin is present in the homozygous condition the polyphenol content in the fruit is also the lowest and also lower than rin present in the heterozygous condition. The reason for this phenomenon could be explained by the failure of climacteric respiration and reduction in evolution of the hormone ethylene (Vrebalov et al., 2002).
Chromatographic representation of carotenoid profiles for the different genotypes observed in progeny is summarized in Figure 3.1. The carotenoid concentrations of individual genotypes with different combinations of \textit{rin} and \textit{t} alleles are listed in Table 3.3 and Table 3.4 respectively. Carotenoid concentration in the progeny ranged from 2.9-33.6 µg/g tetra-cis lycopene, 0-20.5 µg/g ζ-carotene, 0-8.8 µg/g β-carotene, 0-5.5 µg/g neurosporene, and 2.2-19.2 µg/g phytoene (Table 3.3). When \textit{rin} is present in the homozygous condition, the carotenoid concentration is significantly lower (P <0.0001) with a 91% decrease in the predominant carotenoid, tetra-cis lycopene. When \textit{rin} is in the heterozygous condition there is a 45% decrease in tetra-cis lycopene compared to the \textit{wt}. A slight increase in β-carotene was observed when the \textit{rin} allele was present in either the homozygous or heterozygous condition, with the homozygous having significantly more β-carotene than the heterozygous (P <0.0001). Carotenoid precursors, ζ-carotene, neurosporene, and phytoene, decreased 42%, 49%, and 51% respectively relative to the \textit{wt} when \textit{rin} was in the heterozygous condition (P < 0.0001). No precursors, ζ-carotene or neurosporene were detected when \textit{rin} was present in the homozygous condition and phytoene decreased 88% with respect to the \textit{wt} (P <0.0001). Although the \textit{rin} mutation is not directly involved in carotenoid biosynthesis, ANOVA showed that it explained 68-84% of the variation in total carotenoid concentration while environment and uncontrolled error explained the rest. These results are consistent with the results obtained from the parents and hybrids used to develop the segregating population; fruits with the \textit{rin} background had significantly lower carotenoids than \textit{wt} fruits.
Carotenoid concentrations for individuals differing in the allele at the tangerine locus are summarized in Table 3.4. Levels of carotenoids ranged from 14.3-22.4 µg/g tetra-cis lycopene, 11.2-13.0 µg/g ζ-carotenes, 0-4.4 µg/g β-carotene, 2.8-5.4 µg/g neurosporene, and 9.7-12.5 µg/g phytoene. Individuals carrying both alleles (t and t′) had a decrease in tetra-cis lycopene of 43% with respect to those carrying the t′ allele, and a 57% increase in tetra-cis lycopene with respect to the t allele. The concentration of β-carotene was also higher for the individuals homozygous for t than for the heterozygote class. For individuals homozygous for the t′ allele, no β-carotene was detected. The differences in ζ-carotene, neurosporene, and phytoene were not significantly affected by the allele present at the tangerine locus. These results suggest that the state of the locus, whether it is t, t′ or heterozygous, does not significantly affect the concentration of tetra-cis lycopene precursors but tetra-cis lycopene itself.

In conclusion, fruits in the rin background fail to accumulate chromoplast pigments probably due to the lack of ethylene sensitivity and corresponding reduction in ripening (Vrebalov et al., 2002). Plants that are heterozygous for rin have a higher accumulation of carotenoids compared to the homozygotes, suggesting additive to recessive gene action. Explicitly, when plants are heterozygous for rin the ripening and maturation process continues, but at a reduced pace relative to wild type plants. The use of rin by plant breeders to develop long shelf life tomatoes, may decrease fruit quality with respect to nutrients and health promoting carotenoids. Our results also showed that differences in carotenoid concentration attributed to alleles at the tangerine locus only affected the concentration of tetra-cis lycopene and not on the other carotenoids in our
populations. This study shows that the concentration of carotenoids in tomato fruits is highly affected by the genetic background of the plant.

<table>
<thead>
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<th>Marker</th>
<th>Sequence</th>
<th>Gene</th>
<th>Marker type</th>
<th>Temp (°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
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<td>5'-ATACGATAATGTACAACCCGAAAATG-3'</td>
<td>Rin</td>
<td>Size pm</td>
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<td>44</td>
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<td>Rin</td>
<td>Size pm</td>
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<td>44</td>
</tr>
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<td>44</td>
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<tr>
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<td>rin</td>
<td>Size pm</td>
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<td>44</td>
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<td>t</td>
<td>Size pm</td>
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<tr>
<td>TangedelR</td>
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<td>t</td>
<td>Size pm</td>
<td>52</td>
<td>44</td>
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</table>

Table 3.1. List of primers used as markers to detect the presence of genes affecting ripening and carotenoid structure. 1Rin, ripening inhibitor codes for the gene LeMADS-RIN which regulates ripening in tomato fruits; rin, recessive ripening inhibitor mutation that leads to the inhibition of all ripening phenomena; t, tangerine codes for a non functional carotenoid isomerase (CRTISO), this mutation in tomato leads to the accumulation of tetra-cis lycopene at the expense of trans-lycopene. 2 Size pm, size polymorphism.
Figure 3.1. HPLC separation of the carotenoids present in tomato fruits from selected genotypes combining genes and alleles that affect carotenoid structure (t) and genes affecting fruit ripening (rin). Tangerine (t) codes for a non functional carotenoid isomerase (CRTISO) and is an allele of tangerine virescent (t'), the presence of these alleles leads to the accumulation of tetra-cis lycopene instead of trans-lycopene; rin, recessive ripening inhibitor that codes for a chimeric RNA for the gene LeMADS-RIN, this mutation causes the inhibition of all ripening phenomena in tomato fruits; wt, wild type. Peak identification: β-carotene (1), lycopene (2), tetra-cis lycopene (3), phytoene (4), ζ-carotene (5), and neurosporene (6).
<table>
<thead>
<tr>
<th>Lines/Varieties</th>
<th>Genotype</th>
<th>µg/g fresh fruit</th>
<th>cis-lycopene isomers</th>
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<td></td>
<td></td>
<td>tetra-cis lycopene</td>
<td>ζ-carotene</td>
</tr>
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<td>Carolina gold</td>
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</tr>
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<td>02-1007-3</td>
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<td>12.7</td>
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<td>NC99471-4</td>
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<tr>
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<td>P-value</td>
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<td>&lt;0.0001</td>
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Table 3.2. Mean comparison of carotenoid concentration in fruits of the different tomato lines/varieties used as parents for developing segregating rin x t populations. ¹Lines are indicated by Ohio accession numbers or variety name and NC accession number for lines and varieties obtained from North Carolina State University. ²t, the tangerine gene codes for a non functional carotenoid isomerase (CRTISO) and is an allele of tangerine virescent (t'), the presence of this gene in tomato leads in the accumulation of tetra-cis lycopene instead of trans-lycopene; rin, recessive ripening inhibitor that codes for a chimeric RNA for the gene LeMADS-RIN, this mutation causes the inhibition of all ripening phenomena in tomato fruits; wt, wild type. ³Quantities are expressed in µg/g of ripe fresh fruit. ⁴Quantification was based on ζ-carotene standard curve. ⁵Quantification was based on tetra-cis lycopene standard curve. Values entered as “0” were below the level of detection.
Table 3.3. The presence of *rin* in the homozygous condition significantly altered the carotenoid concentration.  

<table>
<thead>
<tr>
<th>Genotype</th>
<th>μg/g fresh fruit</th>
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<td>rin</td>
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<tr>
<td></td>
<td>tetra-cis lycopene</td>
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<tr>
<td>rin</td>
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<td>het</td>
<td>0</td>
</tr>
<tr>
<td>wt</td>
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<tr>
<td></td>
<td>ζ-carotene</td>
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<tr>
<td>rin</td>
<td>0</td>
</tr>
<tr>
<td>het</td>
<td>8.8</td>
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<tr>
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Table 3.4. Comparison of carotenoid concentration for individuals within *t* genotype.  

<table>
<thead>
<tr>
<th>Genotype</th>
<th>μg/g fresh fruit</th>
</tr>
</thead>
<tbody>
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<td>het</td>
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</tr>
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REFERENCES


CHAPTER 4

Purple heirloom tomatoes (*Lycopersicon esculentum* Mill.) retain chlorophyll due to the presence of the *gf* allele

ABSTRACT

Tomatoes with flesh color described as “purple” and “black” are of interest due to increased emphasis on plant pigments and human health, but these varieties have not been subjected to rigorous analysis and the biochemical basis of the color is not known. This work aimed to study the phytochemistry of purple heirloom tomatoes as a potential source of increased dietary pigments. We tested two specific hypotheses concerning the basis of the purple pigmentation, increase of poly-phenols and retention of chlorophyll. Populations were developed by crossing heirloom varieties described as “purple” and “black” to processing tomato breeding lines, followed by self fertilization to develop segregating populations. The resulting F2 populations segregated for a range of pigment types and colors. HPLC traces showed that populations ranged in lycopene concentration from 11-108 µg/g, β-carotene from 3-7 µg/g, and tetra-cis lycopene concentration from 6-237 µg/g on a fresh weight basis. An assay of total phenolics demonstrated that purple heirloom tomatoes do not have higher amounts of poly-phenol compounds. The
heirloom varieties tested did retain chlorophyll after ripening. Allelism test-crosses between purple heirloom varieties and genetic stocks carrying the green flesh (gf) gene resulted in all hybrids having the purple phenotype. These results suggest that the gf mutation is responsible for the purple phenotype, and that the purple color in the heirloom varieties we studied is due to the retention of chlorophyll as the plastid accumulates lycopene.

INTRODUCTION

There is a growing scientific interest in plant secondary metabolites due to their potential health promoting properties (Gómez-Prieto et al., 2003). Carotenoids and flavonoids have warranted scientific attention due to their antioxidant capacity, and correlative evidence linking consumption with reductions in certain chronic diseases (Verhoeyen et al., 2002; Clinton, 1998; Nguyen and Schwartz, 1998). Oxidative damage has been linked to cancer and heart disease, the two leading causes of death in the United States (Nelson et al., 2003; Quinn et al. 2003). Therefore correlative evidence supports current interest in food products that may play a role in reducing incidence of cancer and heart disease.

Another interesting role of carotenoids in human nutrition is the pro-vitamin A property of β-carotene. This carotenoid is of dietary significance due to the incidence of vitamin A deficiencies in developing countries, primarily affecting preschool children, and women of reproductive age (WHO/UNICEF, 1995). A scarcity of vitamin A can
lead to blindness while complete deficiency can lead to premature embryonic death (Kiefer et al., 2002). Consequently, carotenoids can also provide necessary nutrients to the human diet.

Poly-phenols are also of interest due to their bioactivity in many natural processes and due to correlative evidence that their consumption may improve human health. A well studied group of poly-phenols are the flavonoids which are involved in several processes in plants such as pigmentation, resistance to pathogens, seed development, pollen growth, response to nutrient deficiency, UV light protection, and inhibition of growth when plants are in close proximity. Flavonoids also serve as signals, mediating beneficial and pathogenic host-microbe interactions by the induction of microbial genes such as the nod genes in *Rhizobium* and the vir genes in *Agrobacterium* (Spencer and Towers, 1988; Peters et al., 1986; Maxwell et al., 1990). As a constituent of the human diet, flavonoids may serve an important role. Epidemiological research suggests a direct correlation between high flavonoid intake and the reduction of risk of heart disease and cancer (Miean and Mohamed, 2001). A study conducted by Wang et al. (2003) supported the association of flavonoids with the reduction of risk of cancer by inhibiting the insulin-like growth factor-I (IGF-I) signaling cascades. Flavonoids, in particular flavonols (kaempferol and quercetin), have been shown to have antioxidant capacity (Verhoeyen et al., 2002), suggesting a possible mechanism for health promoting properties.

Heirloom tomatoes having fruit described as “purple” and “black” are of interest due to their potential to contain novel pigments. However these varieties have not been subjected to careful examination, and competing hypotheses may be proposed to explain
their color. Increased flavonoid pigments in tomato have been documented in crosses that incorporate the *Anthocyanin fruit* (*Aft*) gene from wild species and genetic stocks (Jones et al., 2003), and it is possible that the purple color in heirloom varieties is due to phenolic pigments. A superimposition of green chlorophyll and red lycopene may also result in a purple color. In the *gf* mutant of tomato, the chlorophyll degradation pathway is altered. Chlorophyll, thylakoids and some thylakoid proteins are retained in the plastid as it accumulates lycopene (Akhtar et al., 1999). This irregularity is caused by a mutation that alters the normal evolution of chloroplast to chromoplast giving to *gf* fruit their characteristic purple-brownish color (Cheung et al., 1993). Regardless of competing hypothesis explaining the purple color in heirloom tomatoes, these tomatoes may provide a source of dietary pigments resulting from the attenuated degradation of chlorophyll or increased flavonoids. The aim of this work was to study the phytochemistry of “purple” heirloom tomatoes as a potential source of increased fruit pigments.

**MATERIALS AND METHODS**

*Plant material and population design.* Tomato populations were developed by crossing purple heirloom varieties (‘Black’, ‘Black Plumb’ and ‘Black Prince’) to Ohio breeding lines. Specifically, Black x Ohio 99-5810, Black Plumb x Ohio 99-5810, and Black Prince x Ohio 99-5807 were studied. Hybrids were grown in the greenhouse and self-fertilized to produce three segregating F2 populations. Individual F2 plants were grown in the greenhouse and were self-fertilized to produce F3 families for replicated testing in the field. Both Ohio lines contained the tangerine allele, *t*, and populations
therefore segregated for “purple fruit” in a red background and in a tangerine background.

An allelism test was conducted between heirloom varieties, ‘Black’, ‘Black Plumb’, ‘Black Prince’, ‘Purple Calabas’ and ‘Black from Tula’, by crossing to *green flesh* genetic stocks, accessions LA2999 and LA3534, provided by the Tomato Genetic Resource Center, University of California, Davis.

**Greenhouse and field growth conditions.** F₂ populations were seeded into 288-well flats containing Metro Mix 360 soil media (Premier Horticulture Inc., Red Hill, PA). Plants were grown in a greenhouse with a day temperature of 23.8° ±3°C and a night temperature of 18.3° ±3°C with a 14/10 h light/dark period. Supplemental high-intensity discharge lighting was provided when natural light fell below 200 W/m². Seedlings were fertilized twice per day with 20 ppm N-P-K (Peters Professional All-Purpose Fertilizer, 20-20-20, Scotts-Sierra Horticultural Products Co., Marysville, OH). Seedlings were transplanted into 10 cM pots, and allowed to self fertilize. F₃ families were seeded into 288-well flats containing the same soil mix and greenhouse conditions as described above and then planted to the field at the Horticulture and Crop Science Farm, Wooster, OH following the procedures described in the Ohio Vegetable Production Guide (Precheur, 2000). Families were grown in two completely randomized blocks.

**Phenotypic evaluation.** Individual plants from F₂ populations were evaluated in the greenhouse. Every plant was assayed for chlorophyll retention in leaves (as described by Akhtar et al., 1999), fruits were evaluated for purple color associated with the heirloom parents, and tangerine color associated with the Ohio breeding lines. F₃ families
were analyzed for segregation so that genetic analysis based on phenotype could reflect homozygous and heterozygous classes.

**Genomic DNA extraction.** Tomato leaflets were placed in the bottom of labeled 1.5 ml eppendorf tubes and stored at -80 °C until DNA extraction. Extraction followed the CTAB method (Kabelka et al., 2002) from single leaflets ground in a 200 µl of 1 part of Nuclei Lysis buffer (0.2 M Tris, 0.05 M EDTA, 2.0 M NaCl, 2% CTAB, pH 7.5), 0.4 part 5% sarkosyl, and 1 part DNA extraction buffer (0.35 M Sorbitol, 0.1 M Tris, 0.005 M EDTA, pH 7.5, 3.8 g/1 NaBisulfite added just before use). After samples were ground, an additional 450 µl of buffer mix was added to samples, lids closed, and samples were mixed well by inverting several times. Samples were incubated at 65°C for 20-60 minutes, followed by extraction using 600 µL of chloroform. Aqueous and organic phases were separated by centrifugation for 5 minutes at 8000 x g. DNA was precipitated from the aqueous phase by addition of 650 µl of isopropanol followed by centrifugation for 5 min at 8000 x g. Tubes were allowed to air dry upside down on paper towels. Pellets containing DNA were dissolved in 200 µl of T1/10 E buffer (10mM Tris-HCl, pH 8.0, and 0.1 mM EDTA, pH 8.0).

**Polymerase Chain Reaction (PCR) assay for genotyping.** PCR reactions were conducted in a 20µl reaction volume. Each reaction consisted of 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM MgCl₂, 100 µM of each dNTP, 10 nM IRD 700 Dye-labeled M-13 forward primer (LI-COR, Lincoln, Nebraska), 0.1 µM of M-13 tailed forward primer, 0.1 µM of reverse primer, 2 µl genomic DNA template (5 ng
per reaction), 5% (v/v) Dimethyl sulfoxide (DMSO), and 1 unit of Taq DNA polymerase. Reactions were heated at 94 °C for 2 min, followed by 36 cycles of 45-s at 94 °C, 45-s at 50 °C, and a 45-s extension at 72 °C. Final reactions were extended at 72 °C for 5 min. Amplification was performed in a PTC-100™ programmable Thermal Controller (MJ Research, Inc. Watertown, MA). Following the amplification reactions, 14 µl of the amplification reaction products were mixed with 3 µl of formamide loading buffer (95% formamide, 20 mM EDTA pH 8.0, and bromo phenol blue), denatured at 95°C for 3 min, and then chilled immediately to 4 °C. Each sample, 0.8 µl, was loaded onto a 7% denaturing gel (7.0 M Urea, 7% acrylamide, 1×TBE) using an 8-channel Hamilton syringe (Hamilton Company, Reno, NV). IRD-labeled molecular markers (Lincoln, Nebraska) were loaded in two-end lanes as a standard. Electrophoresis was performed at constant power (40 W). Data were collected using the Saga Generation 2 software package (Lincoln, Nebraska). Automated band calling and size determination were performed using the same software. The sequences of the primers used as a molecular marker for chromosome 8 were:

5'-CACGACGTTGTAAAACGACCCAAATA A TCCATCTCA-3' for SSR63 Forward and 5' -GCTCCGCCATACTGATACG-3' for SSR63 Reverse.

**Extraction and quantification of chlorophyll.** The method used by Kleinhenz et al. (2003) was modified for chlorophyll extraction from tomato fruit. Eppendorf tubes containing 0.5 g of blended fruit were mixed with 1 ml of ether to extract the chlorophyll (extraction 1:2), and then agitated for about 5 seconds. Samples were incubated in the dark at 4 °C for 1 hour with occasionally agitation. After the extraction period, samples
were centrifuged using a Fisher scientific, Marathon 16 km micro-centrifuge at 9300 x g for 10 min to remove remaining solids. Supernatants were then transferred to fresh eppendorf tubes. Samples were kept on ice all the time to prevent ether evaporation. Readings of absorbance at 660 nm (chlorophyll a (ChlA)) and 642 nm (chlorophyll b (ChlB)) were taken using a DU® 640 Beckman spectrophotometer. A standard curve was developed to determine the relationship between known concentration of chlorophyll and absorbance readings. A new standard curve was prepare each time chlorophyll was measured.

**Carotenoid extraction.** Carotenoid extraction was performed using a modified version of the hexane extraction methods described by Perruzzi et al., 1998. Briefly, five grams of pureed tomato were weighed on an analytical scale, and 1.0g of calcium carbonate (Precipitated chalk, Fisher) and 4.0g of celite (Celite 545, Fisher) were added. Fifty ml of methanol was added followed by further stirring. The mixture was allowed to stand for approximately 1 min followed by homogenization for 1 min. The methanol extract was filtered using Whatman paper no. 1 and 42 (42 on the bottom), pre wet in methanol. The filtrate was suspended in 50 ml of 1:1 acetone/hexane and allowed to stand for 1 min prior to homogenization. The extract was filtered as before and the filtrate saved. Repeated acetone/hexane extractions (up to three times) were required to recover the majority of lycopene. The liquid phase was pooled, transferred to a separatory funnel and washed with approximately 20 ml of distilled water. The bottom layer (aqueous) was discarded and the rinsing step repeated for the top layer (organic). The top layer (organic) was filtered through sodium sulfate (Sodium sulfate anhydrous, Fisher) and glass wool to
remove remaining water, and rinsed with hexane. The final volume was made up to 100 ml with hexane. Five vials with aliquots (3.0 ml) of the diluted extract (extract was diluted 1/5 in hexane) were dried under nitrogen to remove the solvent. The vials were wrapped in aluminum foil to protect from light and placed at –20°C until HPLC analysis.

**HPLC separation and carotenoid and chlorophyll quantification.** The chromatographic separations of carotenoids were performed using a VYDAC™ reversed-phase C18 column on a Waters 2695 HPLC. Dried extracts were reconstituted with appropriate volumes (3 ml) of MTBE (ether) and methanol (1:2) respectively. Samples of 100 µl were injected to a linear gradient (0 min, 100% of 1% of Ammonium Acetate in MeOH; at 20 min, 80% of 1% of ammonium acetate in MeOH and 20% MTBE; at 25 min, 80% of 1% of ammonium acetate in MeOH and 20% MTBE; at 30 min, 100% of 1% of ammonium acetate in MeOH) with a flow rate of 1 ml/min for 30 min to separate carotenoid pigments. Spectral information from 210-700 nm was recorded using a Waters 996 photo-diode array detector. Carotenoids were identified by their characteristic absorption spectra in comparison to carotenoid standards. The quantity of each carotenoid was determined by the integration of the peak area using the Empower software (Waters Corporation, 2003).

**Generation of standard curves.** Bulk extraction from 20 g of puree from selection FG03-310 (selection carrying tangerine and dark green genes) was performed in order to isolate tetra-cis lycopene. Briefly, the ~75 ml hexane extraction (following the carotenoid extraction described above) was concentrated in a rotavapor (Büchi, R110 rotavapor) at 60 °C to an approximate volume of 10 ml. The concentrated extract was dried under a
stream of compressed nitrogen, and the dried extract was wrapped in aluminum foil to protect from light prior to storage at -20 °C freezer until preparative HPLC separation.

The dried extract was reconstituted in 1 ml of a mixture of MTBE (ether) and methanol (1:2) respectively. Pigments were separated using an hp1050 on a reversed-phase C_{18} Waters prep column (125Å, 10µm, 19 x 300 mm). One ml of MTBE: MeOH (1:2) concentrated pigment was injected to the hp1050 HPLC pump on the linear MTBE (solvent A) and 1% of ammonium acetate in MeOH (solvent B) gradient at a flow rate of 5 ml/min. The gradient was in the following format: at 0 min, 100% solvent B; at 40 min, 80% solvent B and 20% solvent A; at 50 min, 80% solvent B and 20% solvent A; at 60 min, 100% solvent B. Detection of tetra-cis lycopene peak was at 438 nm and elution time of 45 min. Purity of the isolated fraction was confirmed using the Waters HPLC following the analytical procedure described above.

A stock solution with an absorbance of ~ 0.9 of the pigment of interest was prepared. Serial dilutions were made from the stock solution. After making the serial dilutions, absorbance of each point of the curve was measured in order to generate a standard curve. Concentration = (Abs/ε); where Abs is the absorbance measured and ε= molar extinction coefficient of the pigment. Chlorophyll a, lycopene and β-carotene standards were purchased from Sigma Chemical Co. (St. Louis, MO).

**Measurement of total phenolics.** The total phenolics were determined using the Folin-Ciocalteu reagent. Briefly, 3 g of homogenized samples were extracted with 40 ml of acetone: water: acetic acid (70:29.5:0.5v/v) and allowed to stand for 1 h at room temperature followed by filtration through Whatman paper no. 1, and supernatants rinsed
with 10 ml water. Filtrates were concentrated to a volume of 25 ml using a rotary evaporator under partial vacuum at 40 °C, and made up to a total volume of 40 ml with dd water. One milliliter of sample was mixed with 1 ml of Folin-Ciocalteu reagent, and 23 ml dd water. This mixture was allowed to stand for 8 min at room temperature followed by the addition of 10 ml of 7% sodium carbonate solution to each sample and allowed to stand for 2 h at room temperature. Absorbance was measured at 750 nm. The total phenolics concentration was standardized against a gallic acid standard curve. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

**Data analysis.** Data were analyzed using the SAS (version 8.2; SAS institute, Cary, NC) general linear model (GLM) procedure for analysis of variance (ANOVA). The standardization of the measurements of compounds was µg/g on a fresh weight basis for chlorophyll, lycopene, tetra-cis lycopene, and β-carotene. Concentration of total phenolics was based on a gallic acid standard curve; results were expressed as mg/l of gallic acid equivalent. The statistical model used for analysis of parental genotypes was:

\[ Y_{ij} = \mu + G_i + r_j + \epsilon_{ij} \]

Where \( Y_{ij} \) = concentration of compound of a plant grown under the \( i^{th} \) level of the factor G, where G is the term for genotype. The term \( \mu \) is the population mean, \( r_j \) is the effect of replicate and \( \epsilon_{ij} \) = the residual error term.

For marker-trait analysis the model tested was:

\[ Y_{ij} = \mu + M_i + r_j + \epsilon_{ij} \]

Where \( Y_i \) = concentration of compound of a plant grown under the \( i^{th} \) level of the factor M, where M is marker SSR63. The term \( \mu \) is the population mean, \( r_j \) is the effect of
replicate and $\epsilon_{ij} =$ the residual error term. Families were considered random effects.

Utilizing this model, the variance of a variable explained by marker SSR63 was equal to the $r^2$ which provided the portion of the variance explained, such that $r^2 = \frac{V_M}{V_M + V_R + V_E}$. Where $V_M =$ variance explained by marker SSR63, $V_R =$ variance due to technical replication, and $V_E =$ variance due to error. Mean comparison of genotypes within marker SSR63 as well as the difference between parents was performed using the last significant difference (LSD) procedure.

A regression analysis was run between the two chlorophyll quantification procedures (HPLC vs Spectrophotometer).

RESULTS AND DISCUSSION

In order to address two competing hypothesis concerning the biochemical basis of color in heirloom varieties described as “purple” or “black”, we assayed fruit for phenolics and chlorophyll. Table 4.1 shows the results for total phenolics assay performed using the Folin-Ciocalteu reagent. This assay revealed that the purple heirloom varieties do not have increased phenolic concentration when compared to varieties with no purple flesh ($P = 0.6992$), suggesting that the color of these varieties might not be due to increased concentration of phenolic pigments. However, ‘Black Plumb’ had significantly higher amount of phenolic compounds ($P = 0.0158$) compared to Black, Black Prince, and the other varieties with no purple flesh. Addition of chloroform to the acetone: water: acetic acid ($70:29.5:0.5$ v/v) extraction, separated an aqueous phase that
was devoid of colored pigments, suggesting that "purple heirloom" tomatoes lacked anthocyanin pigments such as petunidin which gives Aft fruits their purple color (data not shown). In contrast, spectrums in Figure 4.1 suggest that these heirloom tomatoes retain chlorophyll based on the absorbance peak at 660 nm. It is likely that retention of chlorophyll leads to the characteristic "purple" or "black" phenotype because of a superimposition of green chlorophyll over the red lycopene.

Genetic analysis of the purple trait, based on qualitative phenotypic evaluation of fruit suggest the involvement of the green flesh (gf) gene. A survey of 64 SSR molecular markers revealed a polymorphism between parents for marker SSR63, which is on chromosome 8, suggesting the possible use of this marker to establish a relationship between phenotype in our populations and gf which maps to chromosome 8 (Kerr, 1957). This marker revealed the existence of three alleles, 224, 226, and 234; based on the size of the amplified band (bp). Purple heirloom varieties ‘Black’ and ‘Black Prince’ had allele 234, ‘Black Plumb’ had allele 224, and the Ohio breeding lines had allele 226. Chi-square tests were conducted for each population using fruit phenotype as assessed in the F3 generation and marker genotype. Use of F3 families allowed the classification of homozygous and heterozygous plants. For the cross Black Plumb x Ohio 99-5810, the $\chi^2$ was 19.834, for Black x Ohio 99-5810 the $\chi^2$ was 23.38, and for Black Prince x Ohio 99-5807 the $\chi^2$ was 30.18 suggesting a lack of independent assortment (P <0.001) in all three crosses. Our conclusion is that the phenotype of purple heirloom fruit is due to a locus that maps to chromosome 8. Test crosses of the "purple heirloom" varieties to gf genetic stocks yield hybrids with a gf phenotype further suggesting that purple heirloom tomatoes
contain an allele of gf (Figure 4.2). These genetic observations and the observation that purple heirloom tomatoes retain chlorophyll suggest a role for the green flesh (gf) gene in imparting the purple color.

The evaluation of quantitative measurements for chlorophyll also suggest a role for the gf gene, but these analysis are somewhat ambiguous relative to qualitative analysis. 'Black Plumb' had the lowest concentration of chlorophyll of the three heirloom varieties (P = 0.0001). 'Black' and 'Black Prince' had similar carotenoid and chlorophyll concentration (Table 4.1). Data collected using spectrophotometric assays and data collected using HPLC demonstrated a positive linear correlation (P = <0.0001), but the correlation coefficient was low (r² = 0.588). When marker-trait analysis was conducted between SSR63 and chlorophyll in the fruit, a significant association was detected in two of the three populations for data collected using HPLC (Table 4.2). A significant association was detected in only one of the three populations using the spectrophotometric data (Table 4.2). As a Marker for a quantitative trait locus, SSR63 explained as much as 44% of the genetic variation for chlorophyll concentration in the fruit and as little as 1% of the variation, depending on the population and the method of measuring chlorophyll. The ambiguity of these quantitative analyses may be due to both experimental and genetic causes. Field notes indicate that the uniform ripening (u) gene was segregating in the populations. The wild-type allele of this gene leads to a green shoulder on fruit that disappears as chlorophyll degrades unevenly during the ripening process. Experimental factors affecting these data include progeny measurements that
exceed or were below the range of the standard curves leading to questionable accuracy across the range of measurements.

The chlorophyll biosynthesis pathway has been well elucidated while there is lack of knowledge concerning its degradation. Genes encoding the enzymes mediating the chlorophyll degradation pathway are just beginning to be cloned explaining the poor understanding of this pathway. Several degradation pathways have been proposed. Takamiya et al., 2000, proposed a pathway which comprises two stages and five enzymes, where in the first stage the catabolites are still green while in the second or late stage they are colorless. Ni et al., 2001 proposed an alternative pathway which involves three enzymes in two pathways: the pheophorbide pathway and the oxidative bleaching pathway. Hörtensteiner, 2004 proposed a pathway similar to that of Takamiya et al., 2000, where he actually named the key enzymes: pheide a oxygenase (PaO), and RCC reductase (RCCR). Hörtensteiner, 2004 explained that the key reaction in the chlorophyll degradation pathway is the joint reaction of PaO and RCCR to produce the primary fluorescent chlorophyll catabolite (pFCC) which is then converted to non-fluorescent chlorophyll catabolite (NCC). According to Tsuchiya et al., 1999, the first steps of chlorophyll degradation occur in the chloroplast then the fluorescent catabolites are exported to the cytosol, slightly modified, and then imported into the vacuole.

We were interested in analyzing the concentration of carotenoid pigments in the heirloom varieties because previous studies demonstrate that the gf gene alters chromoplast structure and may alter lycopene content (Akhtar et al., 1999). Concentration of carotenoid pigments in lines and varieties used as parents for
developing the segregating population under study are listed in Table 4.2. Pigment concentration varied from 0-57.1 µg/g lycopene, 0-4.5 µg/g β-carotene, 0-52.8 µg/g tetra-cis lycopene, ‘Black Plumb’ had the highest concentration of lycopene of the three heirloom varieties (P= 0.0001). ‘Black’ and ‘Black Prince’ had similar carotenoid concentration. For analysis of carotenoids in the progeny, we divided the populations into “red” and “tangerine” so as not to confound the statistical analysis of β-carotene, trans-lycopene and cis-lycopene by progeny that contain none of the given class of pigment. The tangerine allele t results in the production of tetra-cis lycopene at the expense of down-stream carotenoids. In the absence of this allele all carotenoids are converted to trans-isomers. In the cross Black Plumb x Ohio 99-5810, carotenoid concentration was significantly associated with SSR63. In the cross Black Prince x Ohio 99-5807, the allele from the Ohio parent contributed to lower carotenoid concentration (Table 4.3). Black and Black Prince had allele 234 while Black Plumb had allele 224 suggesting a possible effect by allele 234 in the increase of carotenoids. However, taken together these results suggest that purple heirloom varieties in general will not be a source of high carotenoid pigments.
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Table 4.1. Comparison of phenols, chlorophyll, and carotenoid concentration of parents used to develop the gfx x i<sup>7</sup> segregating polpulations. ¹Lines are indicated by Ohio accession numbers; Variety names are: ‘Black’, ‘Black Plumb’, and ‘Black Prince’. ²Quantification of total phenolics was based on a Gallic Acid (GA) standard curve, expressed as mg/L GA equivalent. ³Measurement was taken using a spectrophotometer. ⁴Measurement was taken using a HPLC. Values entered as “0” were below the level of detection.
Figure 4.1. Spectrum of different fruit tissues of "purple heirloom" varieties (left) compared to red tomato (right). The presence of a peak at 660 nm in all the figures of purple heirloom varieties suggests the presence of chlorophyll. Tissues are: (A) skin, (B) locule wall, (C) gel, and (D) core.
Table 4.2. Marker-trait analysis of chlorophyll and carotenoid concentration in three populations. 

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<sup>1</sup>SSR63 is a marker on chromosome 8 that is linked to the <em>gf</em> gene; <sup>2</sup>Quantities are expressed in µg/g of ripe fresh fruit. <sup>3</sup>Were measured using HPLC techniques. <sup>4</sup>Measured using a spectrophotometer.

Values entered as “0” were below the level of detection.
Figure 4.2. Crosses of “purple heirloom” varieties with green flesh (gf) genetic stocks yield hybrids with gf phenotype. Green flesh (gf) is mutation on chromosome 8 that alters the normal evolution of chloroplast to chromoplast leading to the retention of chlorophyll as the plastid accumulates carotenoids.
REFERENCES


Carotenoids are compounds of lipid nature and are yellow to red in color. They are derived from phytoene, and are the only group of the tetraterpenes (Packer et al., 1999). Their extended chain with conjugated double bonds is considered to be responsible for their antioxidant activity and color (Stahl and Seis, 1999). Carotenoids protect biological systems by physical quenching of singlet oxygen ($^{1}{\text{O}}_{2}$) where the exited $^{1}{\text{O}}_{2}$ transfers its energy to the carotenoid molecule. Lycopene which contains a straight open-chain and 11 conjugated and 2 non-conjugated double bonds is considered to be the most efficient in the quenching of singlet oxygen (Stahl and Seis, 1999). Clinical nutrition studies have shown a correlation between high intake of red tomato and tomato products, and reduction of risk of certain type of cancers (Clinton, 1998; Nguyen and Schwartz, 1999). The antioxidant capacity of lycopene suggests a possible mechanistic link to reductions in cancer and heart disease, though cause-effect have not been clearly established. Other studies suggest that following a diet rich in carotenoids will reduce the risks of developing certain cancers, chronic diseases, macular degeneration and radiation damage (Hedrén et al., 2002; Fraser et al., 2001; Van
Breemen et al., 2002). Carotenoid precursors may also have a potential as anti-cancer compounds in the diet with ζ-carotene being highly efficient (Nara et al., 2001).

Lykopene exists in alternative forms which can be in the trans or cis configuration. Studies show that when lykopene is ingested in the trans form it is poorly absorbed while the cis configuration is more readily absorbed and probably more bioavailable (Unlu et al., 2003). However, it has not been reported how lykopene structure and concentration affect biological activity. Lack of detailed understanding of the relationship between structure and function limits the use of this compound as a functional food.

Tomato is an excellent model for the modification of carotenoid concentration and content due to the available genetic resources. Tomatoes with lykopene in the cis configuration are found in varieties carrying the tangerine mutation. Isaacson et al., 2002 reported that the reason for the accumulation of tetra-cis lykopene at the expense of trans-lykopene in tangerine tomato was due to the lack of a functional carotenoid isomerase (CRTISO). This enzyme, in the carotenoid biosynthetic pathway, converts tetra-cis lykopene to trans-lykopene. Other mutations that affect the carotenoid biosynthetic pathway include Delta, Beta, and old gold crimson (ogc). Other genes alter the chromoplast structure (green flesh) and the chromoplast quantity (dark green), and thus carotenoid concentration and content. Finally, genes that affect the process of ripening (never ripe, non-ripening, ripening inhibitor, and alcobaça) also affect carotenoid profiles (Tigchelaar et al., 1978).
The exploitation of these resources has not been fully explored for the development of plant resources for testing physiological effects in the diet. The results presented in this work show that a germplasm with a wide range of carotenoid structure and concentration can be developed by combining known genes. The lines range in concentration from 0-132 µg/gm lycopene, 0-52 µg/gm β-carotene, 0-45 µg/gm δ-carotene, 0-52 µg/gm tetra-cis lycopene, 0-45 µg/gm phytoene, and 0-62 µg/gm ζ-carotene on a fresh weight basis. However, simple genetic changes may be accompanied by unanticipated changes in carotenoid profile.

When plants have the *ripening inhibitor* (*rin*) mutation in the genetic background the carotenoid profile is significantly affected. When *rin* was present in the homozygous condition, tetra-cis lycopene, decreased by 91% while a 45% decrease was observed when *rin* was in the heterozygous condition. An small increase in β-carotene was observed when *rin* was present in both the homozygous and heterozygous condition, with the homozygous *rin* lines having significantly more β-carotene than the heterozygous. No ζ-carotene and neurosporene were detected in the homozygous *rin*, and these compounds decreased 42% and 49% respectively, relative to wild type plants when *rin* was in the heterozygous condition. Pytoene decreased 88% and 51% when *rin* was in the homozygous and heterozygous condition respectively, relative to wild type plants.

Alternatively, the allele present at the *tangerine* locus did not have a significant effect on the profile of carotenoid precursors, but the concentration of tetra-cis lycopene and β-carotene did vary, with lines carrying the *t* allele containing more β-carotene and lines carrying the *t'* allele containing more tetra-cis lycopene. Hybrid varieties of tomato that
use rin and alcobaça in the background are in commercial production due to the increased field storage and shelf storage, and shipping capacity of fruit that are heterozygous for these genes (Nguyen et al., 1991). However, my results suggest that the presence of these genes can also be accompanied by a decrease in the fruit quality and health promoting carotenoids.

Additionally, here I am reporting that the color of “purple heirloom” tomatoes (‘Black’, ‘Black Prince’, ‘Black Plumb’, ‘Black from Tula’, and ‘Purple Calabas’) is due to the presence of the green flesh (gf) gene and not to an increase phenolic concentration. A total phenolic assay demonstrated that as group, these tomatoes do not have increased phenolic concentration. An allelism test conducted between “purple heirloom” varieties and gf genetic stocks (accessions LA2999 and LA3534) yield hybrids with the gf phenotype confirming the presence of the gf gene. A molecular marker (SSR63) designed for chromosome 8 revealed linkage to the purple fruit phenotype, consisted with the map position of gf. The carotenoid concentration of the progenies was not affected by gf in a consistent manner, suggesting that the genetic background of populations was a more important determinant.

The germplasm developed in this study will provide the raw material for further use in research to address questions concerning the bioavailability and bioactivity of phytonutrients.
REFERENCES


Anonymous. URL: www. tomato. org


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