

# 5.3 Biotechnology of Food Colorant Production

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### 5.3.1 INTRODUCTION

#### 5.3.1.1 CAROTENOID BIOSYNTHESIS: EXAMPLES OF BIOTECHNOLOGY FOR FOOD COLORANTS

Only in the past two decades has the manipulation of biologically produced pigments been informed by a substantial understanding of biochemistry and gene action. While the biotechnological engineers have some complete sets of genes involved in biosynthetic networks, they still have only rudimentary knowledge of the complex behaviors of the many other components of these systems. From an applied perspective, biotechnologists require control of the metabolic cell factory, but also require control of the supplies and demands of its resource environment. The metabolic infrastructure and the information flow through the process (cybernetics<sup>1</sup> or homeostasis) are being probed with pan-cellular tools such as transcriptomics, proteomics, and metabolomics, and also by new computational approaches such as metabolic flux analysis and flux balance analysis.

Truly integrative approaches, those looking across subsets of 'omics data and across metabolic networks, are still on the research horizon. In the meantime, non-integrative approaches now seek the essential components of metabolism. These include: (1) qualitative catalytic activities of enzymes; (2) quantitative flux through metabolic networks; (3) assembly and dynamics of enzyme complexes onto cellular scaffolding (metabolons); (4) supplies of small molecule cofactors and transport chains; (5) feedback mechanisms at the levels of transcription, translation, and catalysis; and (6) mechanisms of transport, sequestration, and storage (or turnover) of accumulating metabolites.<sup>2</sup> Without complete understanding of the components of metabolism, bio-engineers have been often surprised to find that integrated, complex metabolisms are resistant to manipulation.

This section will present a well-studied pigment biosynthetic system that produces carotenoids and apocarotenoids. We present examples of biochemical genetics and successes in proof-of-concept genetic engineering of pigment accumulation as well as lessons learned from unexpected results. While we focus on carotenogenesis and its manipulation in plants, we also look at a few key experiments in fungal and bacterial systems, where most of the groundwork and applications in metabolic engineering occur. This section is divided into three parts that may have more or



less relevance to particular readers. The first part defines biotechnology and the specific technologies applied to modifying pigment accumulation in plants, the second part relates the essential biochemistry and molecular genetics of carotenoid biosynthesis, and the third part discusses recent applications of the biotechnologies to pigment accumulation in edible plants.

### 5.3.1.2 OTHER PIGMENTS: FLAVONOIDS AND BETALAINS

We have chosen carotenoid biosynthesis as the example system for demonstrating the prospects of biotechnology of food colorants for several reasons. Carotenoid biosynthesis is the second most understood system. Multiple examples of valuable food colorant engineering in fungi, bacteria, and plants have been reported. Finally, carotenogenesis in cereal crops such as maize and rice is the primary focus of our research efforts. Hopefully, we provide the food technologist with a template with which to examine other industrially important pigment systems.

Another well-studied system is flavonoid metabolism, which often focuses on flower coloration, but includes important food colorants such as the anthocyanins. Flavonoid metabolism and biotechnology have been most astutely reviewed.<sup>2-10</sup> Research on the biotechnology of betalains is a remarkably expanding field.<sup>3,11</sup> Betalain researchers demonstrated how progress in genomics and molecular genetics gleaned from more studied systems in conjunction with a phyletic perspective are accelerating the pace of discovery and understanding in new fields.<sup>11</sup> We hope that our examples from carotenoid metabolism and engineering will be informative also to those with interest in these other pigment systems.

For carotenoid biosynthesis, genes for the recognized structural enzymes, catalytic activities and essential co-factors have been partially characterized and defined for a number of model crop plants as well as some niche plants such as citrus, marigold, daffodil, gentian, and others. We focus here on a few crop plants and several food colorants —  $\beta$ -carotene, lycopene, lutein, zeaxanthin, astaxanthin, saffron, and bixin (annatto) — for our examples. A number of excellent recent reviews of plant carotenoid biotechnology have been published from several perspectives including biochemical genetics,<sup>12-16</sup> metabolic engineering,<sup>17-23</sup> socio-politics,<sup>24-26</sup> and health and nutrition.<sup>12,25,27-32</sup> Microbial carotenogenesis is not reviewed here, but the reference section includes several reviews.<sup>33-39</sup>

### 5.3.1.3 GENERAL CONCEPTS AND SPECIFIC TECHNIQUES IN PIGMENT BIOTECHNOLOGY

As a preface to examples of biotechnology directed at carotenoid food colorants, we briefly outline concepts in metabolic engineering and definitions of biotechnology, genetic engineering, and metabolic engineering terms. Specific technologies that will be discussed in the context of carotenoid research are gene isolation and functional testing, color complementation in heterologous systems, gene silencing (sense suppression, antisense, and RNAi), gene over-expression, metabolic engineering of pathway flux, metabolic control analysis (MCA), molecular breeding, gene shuffling, recombinant inbred linkage mapping, marker-assisted selection (MAS),

microsynteny comparison, linkage disequilibrium (LD) mapping (association genetics), and quantitative trait loci (QTL) analysis.

#### 5.3.1.3.1 Colors of Biotechnology

Biotechnology is the application of organisms or organism-derived tools to production and process. Biotechnologies have been classified by the target area of application, for example, green biotechnology is applied science involving manipulation of organisms in agricultural endeavors, red in medical endeavors, white in industrial processes, and blue in aquatic processes.<sup>38</sup>

Natural pigment production for food coloration includes the entire spectrum of biotechnologies. For example, biological production of carotenoid pigments has medical implications because carotenoids are nutritive (pro-vitamin A), antioxidant, and photoprotective. Carotenoids are produced alternately in agricultural systems (plants), industrial bioreactors (bacterial and fungi), and marine systems (cyanobacteria and algae).

Historically, biotechnology was the art and science of production organisms grown in tissue culture. Most biotechnology involves microorganisms. Often the production of secondary metabolites such as most pigments occurs after the growth phase of a bacterial, algal, or fungal culture and the amount of pigment produced is directly dependent on biomass accumulation.

Enhancement of biomass production by controlling the environment and nutrition of the organisms favors the accumulation of metabolites. For example, many environmental factors stimulate the accumulation of carotenoids in microorganisms.<sup>35</sup> Bacteria, filamentous fungi, and algae are limited by low biomass production and complicated growth requirements, while fermentation of yeasts (and other ascomycetes), combined with genetic modification may appear more promising.<sup>40</sup> This section examines methods of increasing carotenoid accumulation that are informed by molecular genetics rather than by culture or environmental conditions. Regulatory and public acceptance of new pigments from non-traditional sources seems to be a major impediment to the use of biotechnologically derived pigments. Nevertheless, biotechnological efforts continue and often include strategies that involve genetic manipulation of microorganisms and crop plants.

General reviews of plant genetic and metabolic engineering,<sup>17,19,20,33,41,42</sup> and specific areas, such as tissue-specific gene expression,<sup>43</sup> membrane transport of substrates,<sup>2,21,44</sup> engineering of transcription factors,<sup>3,20,42</sup> and mathematical modeling of pathway flux<sup>45,46</sup> have been published. A general resource on metabolic engineering<sup>47</sup> has also been recently published.

#### 5.3.1.3.2 Genetic Engineering

Biotechnology may involve the use of genetically altered organisms and has become associated with the field of molecular biology. Genetic engineering is the addition of one or a few genes that have first been modified *in vitro* to the stably inherited chromosomes of an organism. The genes may be from the same species or another species. Genetic engineering implies *in vitro* manipulation of specific DNA sequences

and does not include changes to DNA induced by agricultural selection, applied evolution, molecular-assisted breeding, somaclonal variation, or mutation breeding.

The introduced, modified genes are often called transgenes and the plants are called transgenics. *Genetically modified organism* (GMO) is a term most commonly used for transgenics, but is a misnomer because all partners in human co-evolutionary relationships such as domesticated and bred cultivars are genetically modified by human activity. The distinction made between *in vitro* manipulation of specific DNA sequences (GM) *versus* applied evolution and conventional breeding for allelic variation is not always rational or even clear with respect to environmental and health risks.

Currently all GMOs have novel synthetic DNA sequences or sequences added from other organisms. Introduced genes are intended to add or remove a single function and thus present a value-added trait to an otherwise substantially equivalent product. Crop improvement efforts are now refocusing on targeted modification and then selection of endogenous sequences either directly *in planta* (for example, by a mutation breeding technique called TILLING<sup>48</sup>) or via modification of native sequences *in vitro* followed by re-introduction (for example, by a technique called RNA interference or RNAi<sup>49,50</sup>). Genetic engineering of whole pathways by genetic modification of pan-pathway gene regulators such as transcription factors or likewise inducing rational pleiotropic changes is just beginning.

**Requirements for genetic engineering of plants** — To enhance pigment accumulation, biotechnologists must understand all the factors affecting biosynthesis and accumulation. Success has been achieved despite a lack of understanding, but only by a “poke-and-hope” semi-rational approach. Completely rational, predictive engineering requires a fully characterized initial state of the system, regardless of whether one adheres to a deterministic, hierarchical control-based, or stochastic, complexity-based approach to manipulation. Some required, *a priori* knowledge for engineering is covered in Section 5.3.1.1.

Additional “hardware” requirements for genetic engineering are: (1) a genetic transformation method, (2) gene promoters and other *cis*-acting regulatory elements, and (3) a source of genes with proven function. While great progress has been made toward isolation and characterization of gene functions since the early 1980s, some deficits still exist. For example, researchers still face few choices among tissue-specific promoters, very few tested gene regulatory sequences, and many plants that are not easy to transform. Genomics technologies have produced a surplus of genetic elements and supporting information on their expression and relation to metabolism, which will give metabolic engineers the knowledge and tools they need. Current progress may be limited as the burgeoning science of bioinformatics strives to develop the implements needed to mine the full depths of complex information. Functional characterization (biology) and utilization (technology) of the genetic wealth represent the next wave of development in the post-genomic era.<sup>51,52</sup>

**Sources of genes** — New terminology often accompanies changes of scientific paradigms,<sup>53</sup> and this was so when gene sequences suddenly became more available (and numerous) than phenotypes. Reverse genetics is a phrase coined in the 1980s to indicate genetic studies that started with a gene or protein and pursued the associated phenotype. This was opposite the previous experiences of geneticists who



collected phenotypic behaviors and pursued the underlying genetic bases (forward genetics). While the genomic revolution changed the direction of prevailing scientific endeavors, studying phenotypes with simple genetic underpinnings still has an essential impact on the understanding of gene function and metabolism. For many plants, a wealth of classical genetic information and mutant phenotype characterization became available over the past few hundred years. The pre-existing wealth of mutants in pigment phenotypes (for obvious reasons) is large<sup>16,54–56</sup> and has yet to be fully exploited. The importance of classical genetics and such mutants will be illustrated below for several plants.

Genes often come from gene libraries that are complementary to messenger RNAs (cDNA libraries) from particular tissues such as maize endosperms. Interest is often focused on genes involved in the specialized metabolism of that tissue and developmental time rather than “housekeeping” genes found in all cell types. The polymerase chain reaction (PCR) and specific sequence information about genes from other organisms can often be used to produce PCR primers that allow evolutionarily related fragments of new genes to be isolated from the cDNA of the favored target organism.

Homology does not ensure analogy, so function must be demonstrated for the product of the novel gene. This is most easily done in a heterologous complementation system, for example, in transgenic *E. coli*, where the active, recombinant gene product is produced. Particularly elegant and rapid application of these techniques to isolation and understanding of pigment biosynthesis will be given here for  $\beta$ -carotene, lycopene, zeaxanthin, bixin, and saffron, among others. Nevertheless, expression of active, recombinant enzymes for proof of function and characterization of catalytic activity can be difficult and rate-limiting in gene discovery. Newer, facile systems for proof of function such as VIGS and RNAi that are based on removal of normal gene function from plants offer better but currently species-limited approaches. We will describe applications of such gene knockout technologies in Sections 5.3.2.2 and 5.3.3.

The sequencing of entire genomes of *Arabidopsis* and rice and the gene spaces of other plants such as maize<sup>57</sup> has supplied an enormous wealth of genes to metabolic engineers. Huge efforts are needed to associate these genes with phenotypes (reverse genetics) in a high throughput fashion. Another source of gene functions is map-based cloning. Genes with specific suspected functions are isolated by association of phenotypic traits with a genetic recombination-based statistical map of molecular markers on chromosomes. In a second step, the trait-associated molecular markers are then associated with a physical map of a genome and with genomic DNA clones within a library. Finally, among a few candidate genes on the position-identified genomic clone, homology and testing of function may associate a specific gene with a component of a trait.

For production of transgenics, the organism source of a gene seems to matter. One might think that modification of endogenous sequences is best, but gene silencing mechanisms often turn off highly homologous transgenes. While this phenomenon is a boon to functional analysis and genetic engineering of loss of function,<sup>49</sup> it has been a source of unwanted trait variation and unexpected results in some genetic engineering efforts.

Studies have shown that genes from related species are better performing transgenes.<sup>58</sup> Therefore, for transgenesis as well as for a comparative phyletic understanding, homologous genes from pairs of related organisms such as tomato and potato, or maize and rice, or hops and hemp are most useful. Since gene silencing is a transcript-level phenomenon, similar logic applies to the many duplicate gene functions in plants that may be directed by transgenesis with tissue-specific promoters to developmental stages that are different from their normal range (for example a transgenic shoot-specific isoform expressed in a ripening fruit as an adjunct to the endogenous fruit-specific isoform). We can offer several examples from the carotenoid literature in which a phyletically-framed, comparative model system is necessary and most instructive.

**Control of gene expression** — Promoters are sequences outside the protein coding region of a gene that influence the rate of gene product accumulation. They may be constitutive in developmental time or in space or specific to a particular ontogenic or circadian developmental time and place. For example, the plant virus promoter 35S is strong and always in vegetative tissues of most plants, while the wheat glutelin promoter is strongly expressed during mid-endosperm development in a variety of plants.<sup>59</sup> Specific promoters for more tissues are needed. Synthetic promoters may be engineered. Promoter specificity is an issue for both metabolic control and also for biosafety and containment of transgenic products.

The expression of transgenes is affected by the locus of integration, a phenomenon called *position effect*. For plant transformation techniques described below, genes integrate in various and multiple positions, each having a different level of transcriptional activity. This wide variability among events (an event is a single, independent integration) can be used to select the best metabolically balanced phenotype from a range of expression-level variants. Usually at least one hundred events are needed to find production candidates with near-optimal expression, so efficient transformation becomes paramount. Position effects in pigmentation are easy to score, and we will discuss an example below. Homologous recombination that targets a gene to a specific locus is not yet available in higher plants, but understanding and advancement may come from study of the phenomenon in bryophytes.<sup>60</sup>

Regulatory genes code products, often acting as transcription factors that simultaneously activate or suppress panoplies of functionally related genes. Such regulatory genes have great potential for metabolic control of multiple pathway points in a coordinated fashion. Progress toward understanding and use of regulatory genes for plant metabolic engineering of pigment accumulation has been reported for flavonoid biosynthetic systems and recently reviewed.<sup>3,20</sup> Since little progress has been reported for regulatory genes of carotenoid biosynthesis and no carotenoid-specific transcription factors have yet been characterized, our chosen example of carotenoid biotechnology is deficient in this area.

**Transformation** — Most limiting for genetic manipulation of many plant species is the process of introduction of modified genes into the genome. The three major transformation techniques are: (1) bacterial-mediated,<sup>61,62</sup> (2) biolistic,<sup>62</sup> and (3) biolistic plastidial.<sup>63</sup> Bacterial-mediated transformation requires dipping, vacuum infiltration, or co-cultivation of explanted tissue cultures with an *Agrobacterium* or *Rhizobium* transfectant. Biolistic transformation involves the introduction of

exogenous DNA by coating the DNA onto microprojectiles (tungsten or gold dust) and shooting the particles into the cells with a gun. Both methods are followed by embryogenesis and regeneration of plants if appropriate target tissue was used or by analysis of transient expression where regeneration is not possible. Each method has problems and limitations as well as advantages.

Some methods such as floral dip with *Agrobacterium* are very species-limited (to *Arabidopsis*). Many crops (canola, maize, rice, soybean) have been more recalcitrant to transformation by *Agrobacterium* than others (tomato, tobacco). In biolistic transformation, the chromosomes are damaged by the projectiles, and endogenous DNA repair enzymes use the foreign DNA to repair the damage, resulting in transgene integration events.

Major problems with biolistic transformation are rearrangement and integration of multiple copies of transgenes that are stably inherited as linked units. Rearrangements can lead to the production of spurious coding regions and perhaps unintended proteins. Multiple transgenes often lead to co-suppression by silencing of the gene function in later generations and loss of the added trait. Since *Agrobacterium*-mediated transformation is relatively free of these problems, successful efforts have been made to bring this type of transformation to major crops such as, maize,<sup>64</sup> rice,<sup>65</sup> and soybean.<sup>66,67</sup>

Plastid transformation has many advantages including compartmentalization of the gene products in the plastid; prokaryotic-like genetics allowing multiple genes to be coordinately expressed as one polycistronic messenger; and, for the many plants with uniparental inheritance of plastids, elimination of the transgene and transgene products from pollen.<sup>63</sup> Difficulties with transformation and drawbacks of some methods such as gene silencing have impacted the deployment of crops with altered pigment contents. We will describe examples in Section 5.3.3.3.

### 5.3.1.3.3 Molecular Breeding: Linkage Mapping and Association Genetics

Molecular breeding involves a variety of tools to associate a variation in DNA with a variation in a phenotypic trait. The associations may then be applied to crop improvement. An underlying assumption of the method is that the trait of interest is conditioned by a small number of genes with major qualitative or quantitative effects on the trait. The molecular variant (marker) then becomes predictive of the phenotypic trait and can be applied to the selection of progeny at an early stage in a breeding cycle. Marker-assisted selection (MAS) is the use of the DNA markers for applied evolution of a desired trait in a crop or horticultural variety. Linkage mapping is a prospectus study design that associates molecular markers with traits by detecting patterns in carefully prepared progenies of a bi-parental mating. The associations are based on the frequency of recombination and assortment of markers and traits among the progeny.

Linkage mapping is also called *recombinant inbred mapping*.<sup>68</sup> Linkage maps can often be extended from one related organism to another by maps of microsynteny (the linear conservation of gene order and linkage on chromosomes across species). Related species such as maize, sorghum, and rice<sup>69,70</sup> or tomato and bell pepper<sup>71</sup>



can have extensive regions of conserved gene order. Quantitative trait loci (QTL) mapping associates variations in quantitative traits, e.g., pigment content, with molecular markers on a linkage map.<sup>72</sup> QTL can also be associated with candidate genes if a second round of high-resolution mapping is possible, leading to isolation of the gene associated to the phenotype.

Using high-resolution linkage maps to isolate new genes is called *map-based cloning*. High-resolution mapping depends on having a large number of markers and appropriately inbred parents (near-isogenic lines, NILs) and a large number of progeny. Linkage disequilibrium (LD) mapping can also associate candidate loci with a DNA polymorphism, but takes advantage of retrospection of pre-existing allelic diversity within a germplasm collection. LD is the association of alleles across loci.<sup>73,74</sup> LD mapping is not dependent on known pedigree or a pre-prepared recombinant inbred family. The extent (ranging from about 1 to 100 Kb) of LD varies by population and evolutionary (domestication) history of the germplasm collection and varies locally by chromosomal region.

Determining the local extent of LD sets the number of markers needed to associate a QTL with a polymorphism in a gene. Association genetics requires cost-effective detection of sequence polymorphism over extensive loci, and thus is most suited to crops for which large-scale sequence data are already available.<sup>75</sup> Several examples of quantitative genetics applied to basic research of carotenogenesis are described in Section 5.3.2 and specific applications of linkage analysis, QTL mapping, and LD mapping in maize are discussed in the context of biotechnology and breeding in Section 5.3.3.3.

#### 5.3.1.3.4 Generation of Variation: Mutation Breeding, TILLING, and Directed Evolution

Mutation breeding relies on the generation of mutant loci by the application of DNA-altering treatments to plant propagules such as pollen, ovules, meristems, etc.<sup>54</sup> The treatment is often a mutagenic chemical or radiation. Large numbers of treated plants or asexual propagules are scored for fortuitous trait improvement and selected for use in crop improvement. With heavily mutagenized plants, further sexual mating is often needed to separate the value-added trait from spurious, deleterious mutations.

TILLING (targeted induced local lesions in genes) is a gene-targeted mutation breeding strategy.<sup>48</sup> Starting with information (sequence) about the gene, TILLING associates induced sequence polymorphism with traits. TILLING therefore constitutes reverse genetics (from gene to phenotype). Mutations in a gene are detected in a chemical- or radiation-mutated population. Detection of mismatched DNA duplexes (wild-type with mutant) of gene-specific PCR products identifies targeted genetic variants in pools of genomic DNA. The carefully designed genomic DNA pools from mutated plants are systematically deconstructed to identify the unique mutant plant of origin for each gene variant. Phenotypes of known mutants are then analyzed. Many independent mutations in a particular gene (alleles) can be generated and the plants analyzed. Often the mutations are truncations or missense mutations, so knockout of gene function is common. Therefore, null (loss-of-function) mutants of a particular gene and an allelic series of gene variants can



be isolated and used to investigate gene function or used as phenotypic variants for crop improvement.

Directed evolution of enzymes is an *in vitro* method of generating new genes from old ones.<sup>33,76</sup> Pieces of genes from different species may be shuffled together with a PCR technique to create new combinations of exons with altered or new functions. Error-prone polymerases may be used to generate sequence variation at random. Directed evolution has been successful for evolving enzyme variants that are stable in new environments or have altered substrate selection. Pigments have also been synthesized by using novel biosynthetic enzymes that have been evolved from new combinations of natural genes.<sup>33,77</sup>

Transposon tagging and activation tagging have become important technologies in the cloning and analysis of plant genes.<sup>78,79</sup> The cloning is a two-step process.<sup>80</sup> First, tagging relies on the insertion of a genetic element, an endogenous or transgenic, exogenous transposable element or a promoter or enhancer element that changes gene expression upon fortuitous integration within or near a gene function. Second, the perturbation of a gene function with a known (thus, clonable) genetic element allows rescue of surrounding sequences by inverted PCR; hence the gene is tagged for cloning. Each tagging system involves correlation of a mutant phenotype with the insertion, and so pigment phenotypes are especially amenable to tagging.

### 5.3.1.3.5 Metabolic Engineering

Metabolic engineering may involve the use of transgenes, but goes further by rational manipulation of substrate flow through metabolic networks and the consideration of the cell as a unit of production or cell factory. Metabolic engineering often targets the alteration of gene products that influence substrate flow, for example, over-expression of a gene coding a rate-controlling enzyme to enhance substrate flow into a pathway.

While metabolic engineers traditionally sought the rate-limiting enzyme to unlock flow through a pathway, now they understand that there may be many points of control and feedback with the metabolic network, and seek to empirically determine the dynamics of the interactions between rate controllers and other factors. For example, the sizes of metabolic precursor pools and the catabolism or sequestration of products affect accumulation as well as flux through the pathway.

Metabolic control analysis (MCA) assigns a flux control coefficient (FCC) to each step in the pathway and considers the sum of the coefficients.<sup>81</sup> Competing pathway components may have negative FCCs. To measure FCCs, a variety of experimental techniques including radio isotopomers and pulse chase experiments are necessary in a tissue culture system. Perturbation of the system, for example, with over-expression of various genes can be applied iteratively to understand and optimize product accumulation.

Adjunctive to flux control analysis, other components of metabolism that contribute to product accumulation are needed including: (1) substrate/precursor pool sizes (metabolomics), (2) co-factor capacities (metabolomics), (3) gene expression profiles (transcriptomics and quantitative real-time PCR), (4) protein profiles (pro-



teomics), (5) rates of product catabolism (metabolomics), and (6) regulatory mechanisms (enzymology and transcript profiling). Structural components of the metabolic network must also be considered, for example, subcellular localization, metabolic channeling, metabolon assembly dynamics, and intercellular and membrane substrate transport mechanisms, all of which may be interdependent and require small molecule co-factors. Examples of some of these concepts are available for bacterial and plant carotenogenesis and are presented in Section 5.3.3.6.

## 5.3.2 BIOCHEMISTRY AND GENETICS

### 5.3.2.1 OVERVIEW

For the sake of study, the biosynthesis of carotenoid plant pigments can be divided into parts involving enzymes and their associated activities as listed in Table 5.3.1 and further detailed in Figure 5.3.1 through Figure 5.3.4. Some of the parts have common enzymatic mechanisms and may also be in distinct subcellular compartments such as cytoplasm, endoplasmic reticulum, or plastid thylakoid.

Carotenoids are C<sub>40</sub> polyenes containing extended conjugated double bonds that absorb light and give them color. Condensation of the five-carbon phosphate-activated isoprene (C<sub>5</sub>) into longer carbon chain di-phosphate esters, e.g., C<sub>10</sub>, C<sub>15</sub>, C<sub>30</sub>, and C<sub>40</sub>, gives us the C<sub>40</sub> backbone of the carotenoids. We also discuss the biosynthesis of the metabolic precursors for carotenoid formation, the activated isoprene units (IPPs) because genetic engineering within the precursor pathway (DOXP or MEP) and also within the carotenoid pathway has effects on carotenoid accumulation.

The polyene backbone is subject to further paired hydrogen eliminations (desaturations) and isomeric rearrangement to yield the colored polyene. Cyclization of the ends of the chains produces the carotenes. The carotenes can then be remodeled by catalyses that involve oxygen (hydroxylases, epoxidases, de-epoxidases, and oxygenase cleavage enzymes) and yield xanthophylls, ketocarotenoids, and apocarotenoids. The latter parts of the pathway lead to well-known carotenoids found in foods: lycopene,  $\beta$ -carotene, lutein and zeaxanthin, capsanthin and capsorubin, astaxanthin, and the apocarotenoids, bixin and saffron; and for each of these sections of the metabolic pathways, we will discuss the genes and enzymes and substrates in brief detail. This will give us a foundation to provide examples of genetic and metabolic engineering for food colorants. Figure 5.3.1 shows how all parts of the pathways to and from colored carotenoids are interrelated.

### 5.3.2.2 POOLS OF PRECURSORS: MEP PATHWAY TO IPP AND DMAPP

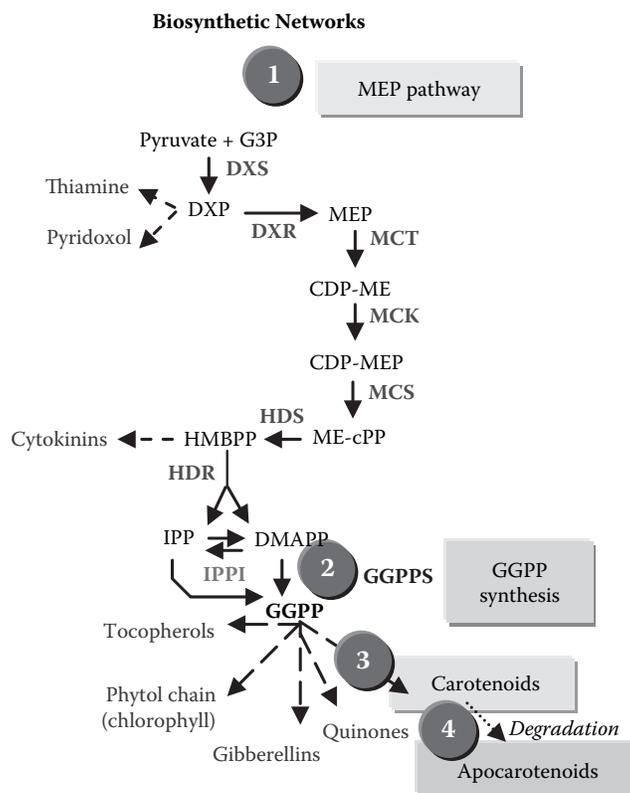
IPP and its DMAPP structural isomer are produced from glycolytic products by the methyl erythritol phosphate (MEP) pathway (Figure 5.3.1, Pathway 1). These isoprene units are condensed in a stepwise fashion to form the precursor to all carotenoids, geranylgeranyl di-phosphate (GGPP). GGPP is not solely metabolized to make carotenoids, but is a precursor for many other primary and secondary metab-

**TABLE 5.3.1**  
**Enzyme Abbreviations and Substrates**

		Enzyme	Name	Substrate
1	MEP Pathway	DXS	1-deoxy-D-xylulose 5-phosphate synthase	D-glyceraldehyde-3-phosphate, pyruvate (G3P)
		DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase	1-deoxy-D-xylulose-5-phosphate (DXP)
		MCT	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase	2C-methyl-D-erythritol-4-phosphate (MEP)
		MCK	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	4-diphosph-2C-methyl-D-erythritol (CDP-ME)
		MCS	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate (CDP-MEP)
		HDS	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	2C-methyl-D-erythritol 2,4-cyclodiphosphate (ME-cPP)
		HDR	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP)
		IPPI	Isopentenyl pyrophosphate isomerase	Dimethylallyl pyrophosphate (DMAPP), isopentenyl pyrophosphate (IPP)
2	GGPPS	GGPPS	Geranylgeranyl pyrophosphate synthase	
3	Carotenoid Biosynthesis	PSY	Phytoene synthase	Geranylgeranyl pyrophosphate (GGPP)
		PDS	Phytoene desaturase	15- <i>cis</i> -phytoene; 9,15- <i>cis</i> -phytofluene
		Z-ISO	15- <i>cis</i> ζ-carotene isomerase	15- <i>cis</i> ζ-carotene
		ZDS	ζ-carotene desaturase	9,9'- <i>cis</i> -ζ-carotene; 9'- <i>cis</i> -neurosporene
		CRTISO	Carotene isomerase	7,9,9'- <i>cis</i> -neurosporene; 7',9'- <i>cis</i> -lycopene
		LCYB	Lycopene β-cyclase	All- <i>trans</i> lycopene; λ-carotene; γ-carotene
		LCYE	Lycopene ε-cyclase	All- <i>trans</i> lycopene
		HYDB	β-ring carotene hydroxylase (diiron type)	β-carotene; β-cryptoxanthin; α-carotene
		CYP97A	β-ring carotene hydroxylase (P450-type)	β-carotene; β-cryptoxanthin; α-carotene
CYP97C	ε-ring carotene hydroxylase (P450-type)	α-carotene, zeinoxanthin		
	ZEP	Zeaxanthin epoxidase	Zeaxanthin; antheraxanthin	

**TABLE 5.3.1 (Continued)**  
**Enzyme Abbreviations and Substrates**

	Enzyme	Name	Substrate	
	VDE	Violaxanthin deepoxidase	Violaxanthin; antheraxanthin	
	NXS	Neoxanthin synthase	Violaxanthin	
4	Carotenoid Degradation	NCED	9- <i>cis</i> -epoxy carotenoid (cleavage) dioxygenase	Various carotenoid substrates (11,12 double bonds)
		CCD	Carotenoid cleavage dioxygenase	Various carotenoid substrates (9,10 double bonds)



**FIGURE 5.3.1** Parts of the isoprenoid pathways to carotenoids. 1 = MEP pathway. 2 = GGPP synthesis. 3 = Carotenoid biosynthetic pathway. 4 = Carotenoid degradative pathways. Enzyme abbreviations and enzyme activities are defined in Table 5.3.1.

olites (Figure 5.3.1, Pathway 2). Two GGPPs are condensed to produce the extended C40 polyene carbon chain that serves as the “backbone” of all carotenoids.

In plant plastids, GGPP is formed from products of glycolysis and is eight enzymatic steps away from “central” glucose metabolism. The MEP pathway (reviewed in recent literature<sup>15,38,82–84</sup>) operates in plastids in plants and is a preferred source (non-mevalonate) of phosphate-activated prenyl units (IPPs) for plastid isoprenoid accumulation, such as the phytol tail of chlorophyll, the backbones of carotenoids, and the cores of monoterpenes such as menthol, linalool, and iridoids, diterpenes such as taxadiene, and the side chains of bioactive prenylated terpenophenolics such as humulone, lupulone, and xanthohumol.<sup>85</sup> The mevalonic pathway to IPP that operates in the cytoplasm is the source of the carbon chains in isoprenes such as the polyisoprene, rubber, and the sesquiterpenes such as caryophyllene.

At the beginning of the MEP pathway, the glycolytic products, pyruvate and D-glyceraldehyde (GAP), are condensed in a transketolase reaction to deoxy-xylulose phosphate (DXP) by the deoxy-xylulose phosphate synthase (DXS) enzyme.<sup>86–88</sup> DXP is the precursor for other pathways leading to pyridoxal and thiamine.<sup>82,89,90</sup> DXP undergoes rearrangement and then is reduced by a reductoisomerase (DXR) to methyl erythritol phosphate (MEP), the first substrate committed to IPP and DMAPP.<sup>91,92</sup>

The genes for *Dxs* and *Dxr* have been cloned from bacteria and plants, including *Arabidopsis*, mint, peppers, marigold (reviewed by Fraser and Bramley<sup>12</sup>), and recently ginkgo.<sup>93</sup> Among three *Dxs* homologues in *Arabidopsis*,<sup>82</sup> only one (*Dxps-2*) has been shown to be functional to date. Other gene families in the carotenoid pathway have photosynthetic (housekeeping) tissue-specific versus storage (secondary metabolite) tissue-specific expression patterns. Both DXS and DXR have been manipulated in bacteria and plants to increase accumulation of pigments. Only DXS seems to be rate-controlling for flux into isoprenoids, although, like DXS, DXR is up-regulated in some plants during isoprenoid accumulation<sup>93</sup> (and other studies have been reviewed<sup>83</sup>).

Recently, a potential cytosolic component of the MEP precursor pathway, xylulose kinase, has been cloned and tested for function in an *Escherichia coli* complementation system.<sup>94</sup> The kinase activates exogenous xylulose in the cytoplasm. DXP is the precursor for DXS, which resides in the plastid, suggesting the activated substrate must be transported into the plastid. Another xylulose kinase homologue in *Arabidopsis* that contains a plastid targeting sequence was not active in the *E. coli* system, suggesting that it may have some other function in the plastid. Perhaps plant and bacterial tissue cultures may be fed xylulose to condition accumulation of isoprenoid metabolites.

MEP is converted to DMAPP and IPP by five more enzymatic steps (Figure 5.3.1), only some of which have been manipulated in plants. Page et al.<sup>95</sup> demonstrated the functions of the latter genes coding enzymes in the metabolic pathway by gene knockout technology. Using viral-induced gene silencing (VIGS), *Hds*, *Hdr*, and *Ippi* functions were knocked out *in planta*. Since interruption of the MEP pathway affects chlorophyll and carotenoid accumulation, the virus-infected leaf tissues were non-pigmented or mottled.

Although exchange of IPP (cross-talk) between cytosols and plastids has been evidenced (cited by Page<sup>95</sup>), the phenotypes of the VIGS knockouts of the MEP pathway show little traffic of MEP metabolites from the cytoplasm into the plastid in *Nicotiana*, and this has been confirmed by others in carrots,<sup>96</sup> *Arabidopsis*,<sup>97</sup> and strawberries,<sup>98</sup> except under certain interesting circumstances reviewed by Rodriguez-Concepcion.<sup>83</sup> *Hdr*, but not *Hds*, was strongly up-regulated at the transcriptional level in de-etiolating *Arabidopsis* leaves and in ripening tomato fruit,<sup>23,99,100</sup> suggesting that HDR is rate-controlling. HDR over-expression studies in plants<sup>100</sup> confirmed earlier studies demonstrating rate-controlling bacterial and plant HDR enzymes expressed in *E. coli*.<sup>101</sup>

Even though HDR produces both DMAPP and IPP and allows some chlorophyll and carotenoids to accumulate in VIGS IPPI knockouts, IPPI seems essential to normal leaf development, and the isomerase may function to adjust the ratio of DMAPP to IPP.<sup>95</sup> Many studies have associated IPPI with degrees of carotenoid pigment accumulation in algae and in heterologous bacterial systems.<sup>101-106</sup> IPPI may be related to cross-talk between the MVA and MEP pathways under certain developmental regimes.<sup>83</sup>

### 5.3.2.3 POLYMERS FROM PRENYLS

GGPP is the head-to-tail condensation product of dimethylallyl di-phosphate (DMAPP) with isopentenyl di-phosphate (IPP). DMAPP is needed as the starting substrate for chain elongation of the polymer. Once produced by HDR (or IPPI), DMAPP is condensed head to tail (1 → 4') with IPP by geranyl di-phosphate synthase (GPPS) to produce GPP (C10). Further condensations of IPP onto the growing polyene by geranylgeranyl di-phosphate synthase (GGPPS) produce in sequence farnesyl di-phosphate (FPP, C15) and geranylgeranyl di-phosphate (GGPP, C20). See Figure 5.3.1, Pathway 2.

These enzymes are substrate-selective and make polymers of discrete chain lengths depending on size and features of their catalytically active pockets.<sup>107</sup> The substrate chain length specificity of GGPPS among various species has been reviewed.<sup>108</sup> Plastidial isoprenoid synthase enzymes have FPS, FPPS, and GGPPS activities. For example, in maize endosperm cytosol, a gene isolated by functional complementation for a GGPPS is a *bone fide* FPPS.<sup>109</sup> Enzymes active in different subcellular compartments are encoded by different genes active in different tissues and may have different substrate preferences. For example, *Arabidopsis* contains five GGPPS genes. Two enzymes are directed to the plastids, two to the cytosol, and one to the mitochondria.<sup>110</sup> Since FPS and GGPPS are responsible for the biosynthesis of quinones, chlorophylls, and carotenoids, they have served as the foci of biotechnology techniques aimed at the accumulation of pigments. Understanding and manipulation are complicated by the many isoforms.

GGPPS functions as part of a complex metabolon. In the plastid, as shown in *Capsicum* chromoplasts,<sup>111</sup> GGPPS is a homodimer and associated but not integral to the plastid envelope. GGPPS is also associated with the next enzyme in the pathway as part of a holoenzyme complex.<sup>112,113</sup>

The first C40 carotenoid, phytoene, is produced by head-to-head condensation of two GGPPs by an enzyme that shares homology to GGPPS and squalene synthases

(sterol biosynthesis). Formation of the C40 isoprene, phytoene, is mediated by phytoene synthase or PSY (reviewed by Cunningham and by Sandmann<sup>114,115</sup>; see Figure 5.3.1, Pathway 3). Phytoene is a colorless carotenoid. PSY mediates a two-step catalysis. First is the head-to-head condensation of two molecules of GGPP to produce pre-phytoene di-phosphate (PPPP). This intermediate is then rearranged to form phytoene as a precursor to all carotenoids.<sup>116</sup> The route of stereo-elimination of hydrogen gives phytoene a central 15-15' Z (*Zusammen*, otherwise named *cis*) double bond (see figure by Pfander<sup>117,118</sup>). The geometric isomer state of the phytoene central Z double bond is an important factor in the accumulation of carotenoids further down the pathway.

Genes coding phytoene synthase have been isolated from many plants; some of the well-studied genes are listed by Fraser.<sup>12</sup> PSY is associated with chaperonin,<sup>119,120</sup> membranes, galactolipids,<sup>121</sup> and GGPPS dimers.<sup>112,113</sup> PSY is rate-controlling for flux into carotenes,<sup>58,122,123</sup> and thus both the plant and bacterial genes have been used for genetic manipulation of carotenoid accumulation (discussed in Section 5.3.3.3).

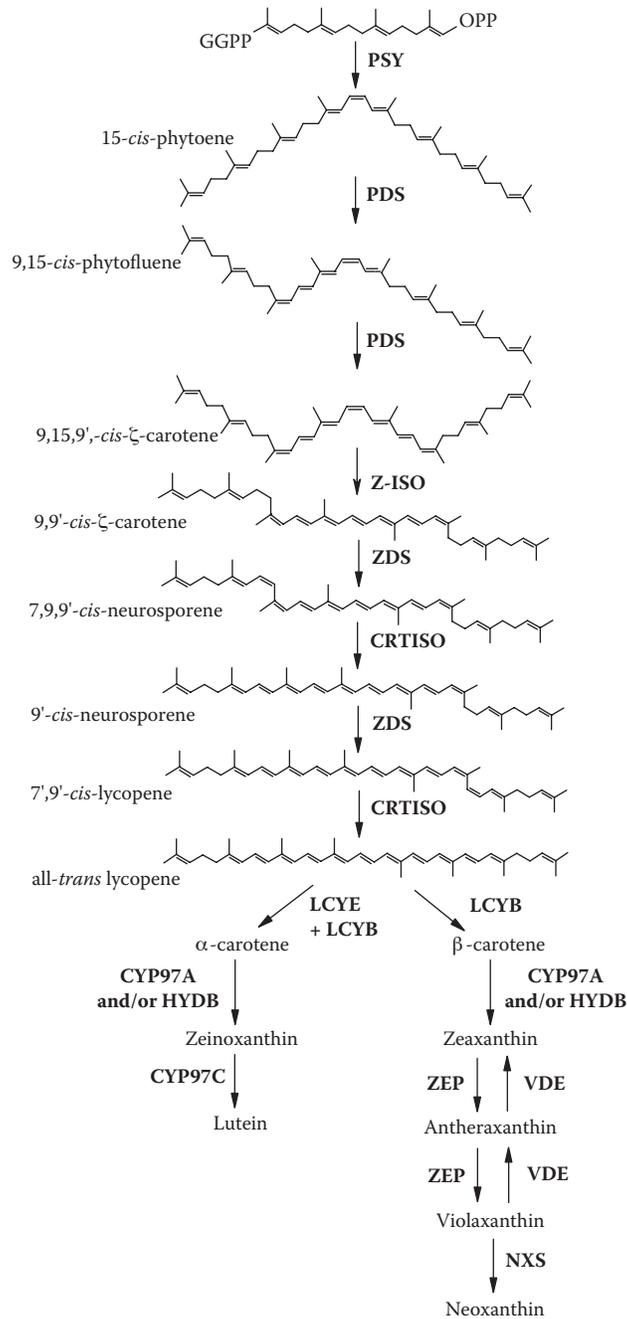
Some plants appear to have only one *Psy* gene.<sup>124</sup> *Psy* gene duplications have been found in tomato,<sup>125,126</sup> tobacco,<sup>127</sup> rice and maize,<sup>118</sup> and other cereal grasses.<sup>128</sup> Maize is an allotetraploid among the grasses (Poaceae),<sup>129</sup> so more than two *Psy* genes might be expected. Interestingly, the duplications of tobacco and tomato are not the same evolutionary event as the duplication of genes in maize and rice.<sup>118</sup> Thus, duplication of *Psy* is an evolutionary parallelism (convergence), having occurred multiple times in plant phylogeny.

PSY is essential for photosynthesis but over-expression in green tissues is detrimental.<sup>130</sup> Evolution of additional *Psy* genes may have allowed high-level expression in other tissues such as roots, anthers, fruits, flowers, and seeds. Thus the co-opting of carotenoids as secondary metabolites involved in animal attraction as pigments or in response to biotic and abiotic stress occurred multiple times during plant evolution. Indeed, in both dicots and monocots, one of the *Psy* duplications has temporal and tissue-specific expression conditioning carotenoids accumulating as secondary metabolites, for example, lycopene in red tomato fruits<sup>113</sup> and yellow xanthophylls in the endosperms of maize.<sup>128</sup> Pigment engineers might take the evolutionary example, and modify the expression of genes involved in secondary metabolite production, without perturbation of primary physiology. The existence of duplicate genes coding stress-inducible or storage tissue-specific isoforms of PSY allows new biotechnological approaches to pigment accumulation.

#### 5.3.2.4 DESATURATION AND ISOMERIZATION TO COLORED CAROTENOIDS: BIOSYNTHESIS OF LYCOPENE

Production of the linear backbone of the carotenes requires four desaturations and several isomerizations of double bonds in the polyene chain (Figure 5.3.2). The substrate of the desaturations, 15-*cis* phytoene, does not accumulate in plants, with the exception of mutants, such as maize *viviparous5*, which has a white kernel and accumulates 15-*cis* isomers of phytoene (unpublished data). Such mutants have and continue to be useful for delineation of the pathway. The desaturations introduce conjugated double bonds extending the double bond system to generate a chro-

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**FIGURE 5.3.2** Carotenoid biosynthetic pathways in plants. Enzyme abbreviations and enzyme activities are defined in Table 5.3.1.

mophore, which changes from yellow to red upon the serial desaturations. Some of the introduced double bonds are *cis*-configured.<sup>131</sup> When lycopene accumulates (e.g., tomato), it is found as the all-*trans* isomer.<sup>132</sup> The change in geometric isomer state is catalyzed by isomerase(s). The isomerization of the *cis*-double bonds to the *trans* configuration is necessary for formation of cyclic carotenes because the downstream lycopene cyclase enzymes are stereospecific for the all-*trans* configuration of lycopene.

In plants, the double bonds are introduced at paired, symmetric positions into 15-*cis* phytoene as shown in Figure 5.3.2. It was thought that only three plant enzymes, phytoene desaturase (PDS),<sup>133</sup>  $\zeta$ -carotene desaturase (ZDS),<sup>134</sup> and carotene isomerase (CRTISO)<sup>135,136</sup> were involved in formation of all-*trans* lycopene. However, recent characterization of the maize *y9* locus has brought to light a new isomerase required in plant carotenoid biosynthesis.<sup>266</sup> Maize *Y9* encodes a factor required for isomerase activity upstream of CRTISO, which has been termed Z-ISO, an activity that catalyzes the *cis* to *trans* conversion of the 15-*cis* bond in 9,15,9'-tri-*cis*- $\zeta$ -carotene, the product of PDS, to form 9,9'-di-*cis*- $\zeta$ -carotene, the substrate of ZDS. Implication of *Y9* as the locus of the novel isomerase was made possible by a step-wise dissection of the carotenoid desaturation pathway in maize by a combination of molecular genetic techniques, including: (1) recombinant inbred gene mapping, (2) RT-PCR analysis of gene expression, (3) chemical complementation, and (4) HPLC analysis of geometric isomer states of pathway intermediates.<sup>145, 201, 266</sup> Careful predictions and observation of accumulation of metabolites and morphological variation in pigment patterns amongst abiotically stressed or light *vs.* dark grown plants was crucial.<sup>266</sup> Thus, identification of this "new" biosynthetic step using a maize mutant particularly well illustrates the value of classical mutant collections.<sup>16</sup>

The two desaturases are membrane-bound enzymes,<sup>111,112,119,137-140</sup> and by inference, the isomerases may be also membrane-bound. Two di-hydrogen eliminations occur at the 11 and 11' positions mediated by PDS and then two more occur at the 7 and 7' positions mediated by ZDS.<sup>141,142</sup> PDS introduces *trans*-configured double bonds at 11 and 11', and is thought to isomerize double bonds at 9 and 9' from *trans* to *cis*. ZDS introduces *cis*-configured double-bonds at the 7 and 7' positions.<sup>143</sup> Only the intermediates, phytofluene and  $\zeta$ -carotene are found among intermediate-accumulating mutants,<sup>144,145</sup> so other sequences of reactions do not occur, for example phytoene is not a substrate for ZDS (no 7,7'-polyene occurs that is not already 11,11' desaturated) and other polyenes, such as 3,4, 3',4' lycopene do not occur naturally in plants, but have been engineered by gene shuffling.<sup>77</sup> Contrary to the activities of PDS and ZDS, in bacteria<sup>146,147</sup> and in some fungi<sup>148-150</sup> three to five serial desaturations of phytoene are carried-out by a single enzyme, the CRTI-type enzyme. Also the microbial CRTIs do not act with step-wise symmetry.<sup>131</sup> Differences in the activities of the bacteria and plant enzymes are important to pigment biotechnology, because each option has been implemented in plant metabolic engineering.

Besides the capacity of CRTI to introduce all four double bonds in the conversion of phytoene to lycopene, the enzyme produces different geometric isomers than does PDS/ZDS (see graphic, side-by-side comparison in Fraser and Bramley<sup>12</sup>). CRTI produces all-*trans* isomers. Studies that have examined the function of the paired plant desaturases acting together, from *Arabidopsis*,<sup>151</sup> and from maize<sup>145</sup> and from

mixed *Synnechocytis*-PDS/tomato-ZDS<sup>143</sup> in heterologous complementation systems, showed that among both the dicot (e.g., tomato and *Arabidopsis*) and monocot (e.g., maize) groups of higher plants, poly *cis*-pathway intermediates and products accumulate. The enzymes produce poly-*cis* lycopene (7,9,7',9'-tetra *cis* lycopene). Mutants that accumulate intermediates of carotenoid biosynthesis in plants also accumulate poly-*cis* isomers<sup>152,153</sup> and methods for detection of isomers of low level intermediates<sup>154</sup> will extend the demonstration of a poly-*cis* pathway to other plants. Since lycopene cyclases (LCY), which produces the cyclic carotenes, are stereoselective for all-*trans* isomers in most systems,<sup>155-159</sup> isomerases are necessary for accumulation of cyclic carotenoids in plants (Figure 5.3.2).

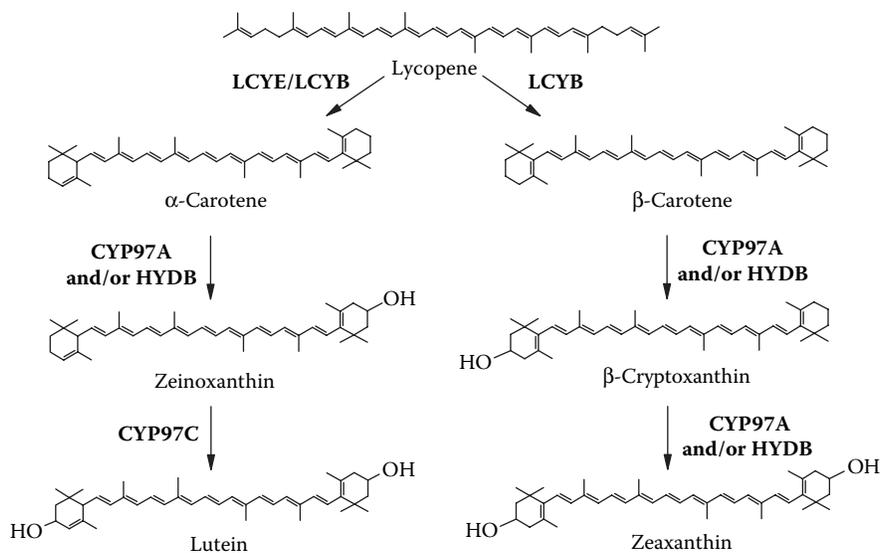
The carotenoid isomerase (*CRTISO*) was the first isomerase associated with the desaturation steps and named at a time when Z-ISO was unknown to exist<sup>136,143,160,161</sup> (and reviewed in references<sup>12,162,163</sup>). *In vitro* analysis of substrate conversion<sup>143</sup> and transcript profiling *in planta*<sup>164</sup> associated *CRTISO* with the desaturation steps. Isaacson demonstrated that *CRTISO* is specific for the 7,9 or 7',9'-*cis* bond configuration and is not involved in the isomerization of the 15-15'-*cis* double bond to the *trans* conformation. As recently shown, Z-ISO is required for isomerization of the 15-15' *cis* double bond of phytoene produced in dark-grown tissues as well as in stressed photosynthetic tissues.<sup>266</sup> Therefore, desaturation of phytoene to lycopene involves a two-step desaturation by PDS, followed by 15-*cis* isomerization by Z-ISO, and then each pair of double bonds introduced by ZDS is followed by *CRTISO*-mediated isomerization of the resulting conjugated double bond pair.

Light may photoisomerize *cis*-carotenoids to the *trans*-states, especially the central 15-15' *cis*-double bond of phytoene, phytofluene, or zetacarotene.<sup>151</sup> In the absence of light, *Arabidopsis* null mutants in *Crtiso* accumulate poly-*cis* lycopene<sup>136</sup> and maize *y9* mutants accumulate the Z-ISO *cis* isomer substrate, 9,15,9'-tri-*cis*- $\zeta$ -carotene.<sup>266</sup> However, on the basis of mutant phenotypes (as discussed<sup>266</sup>), light appears to only partially compensate for lesions in the Z-ISO mutant. Interestingly in *Citrus*, there was no apparent induction of *Crtiso* during fruit ripening, while other genes coding enzymes in the pathway were up-regulated.<sup>164</sup> The genetic identification of Z-ISO will similarly lead to gene isolation, at which point analysis of Z-ISO gene expression during carotenogenesis will be feasible.

### 5.3.2.5 RINGING ENDS: BIOSYNTHESIS OF $\beta$ -CAROTENE

Lycopene is the typical substrate for cyclization. One or both ends of the acyclic precursor can be cyclized. Cyclization can occur in one of two ways to create two different ring structures, differing only by the position of the double bond in the cyclohexane ring. Different enzymes form each of the rings, the lycopene- $\beta$ -cyclase (LCYB) and the lycopene- $\epsilon$ -cyclase (LCYE), as shown in Figure 5.3.3.

While LCYB can use a linear (lycopene) or monocyclic substrate ( $\delta$ -carotene or  $\gamma$ -carotene) to make a symmetric carotene with identical  $\beta$  rings on each end, LCYE introduces only one  $\epsilon$ -ring into lycopene. A notable exception is the lettuce LCYE producing lactucaxanthin, which has two  $\epsilon$ -rings.<sup>165</sup> The pathway bifurcates to form either  $\beta$ -carotene (having two  $\beta$ -rings) or  $\alpha$ -carotene (having one  $\beta$ -ring and one  $\epsilon$ -ring). The relative level of  $\epsilon$ -cyclase activity influences the proportions of  $\alpha$ -



**FIGURE 5.3.3A** Carotenoid cyclization. Enzyme abbreviations and enzyme activities are defined in Table 5.3.1.

*Continued.*

and  $\beta$ -carotene and their downstream oxygenated derivatives, the xanthophylls, lutein, and zeaxanthin.

Cunningham proposed a regulatory scheme by which the stoichiometry of the enzymes dictates the prevalence of alternate metabolons and thus the distribution of the pathway end products.<sup>156</sup> Because of the importance of lutein and zeaxanthin in the photosynthetic apparatus and in the xanthophyll cycle,<sup>166</sup> which mitigates photo-oxidative stress, manipulation of flux through this branch point may allow engineering of photo-oxidative stress tolerance.<sup>167,168</sup> Additionally, since  $\beta$ -carotene is the preferred food colorant and has twice the pro-vitamin A activity of  $\alpha$ -carotene, this branch point is also a target for genetic engineering of crops. Similarly, the accumulation of xanthophylls, ketocarotenoids, and apocarotenoids used as food pigments is also affected by this pathway branch point.

### 5.3.2.6 HYDROXYLATION OF CAROTENES: BIOSYNTHESIS OF LUTEIN AND ZEAXANTHIN

Replacement of the hydrogen at the 3 or 3' position of the carotene ring with a hydroxyl is the next step in both branches of the pathway. Hydroxylation of the rings of the carotenes leads to biosynthesis of the xanthophylls, including the well-known lutein and zeaxanthin food pigments. Lutein is formed by hydroxylation of  $\alpha$ -carotene; zeaxanthin is formed by hydroxylation of  $\beta$ -carotene.

Different monooxygenase enzymes hydroxylate the 3 position of the  $\beta$ - and  $\epsilon$ -rings of  $\alpha$ -carotene. Hydroxylation of one ring of  $\beta$ -carotene produces  $\beta$ -cryptoxanthin and hydroxylation of both  $\beta$ -rings produces zeaxanthin. Hydroxylation

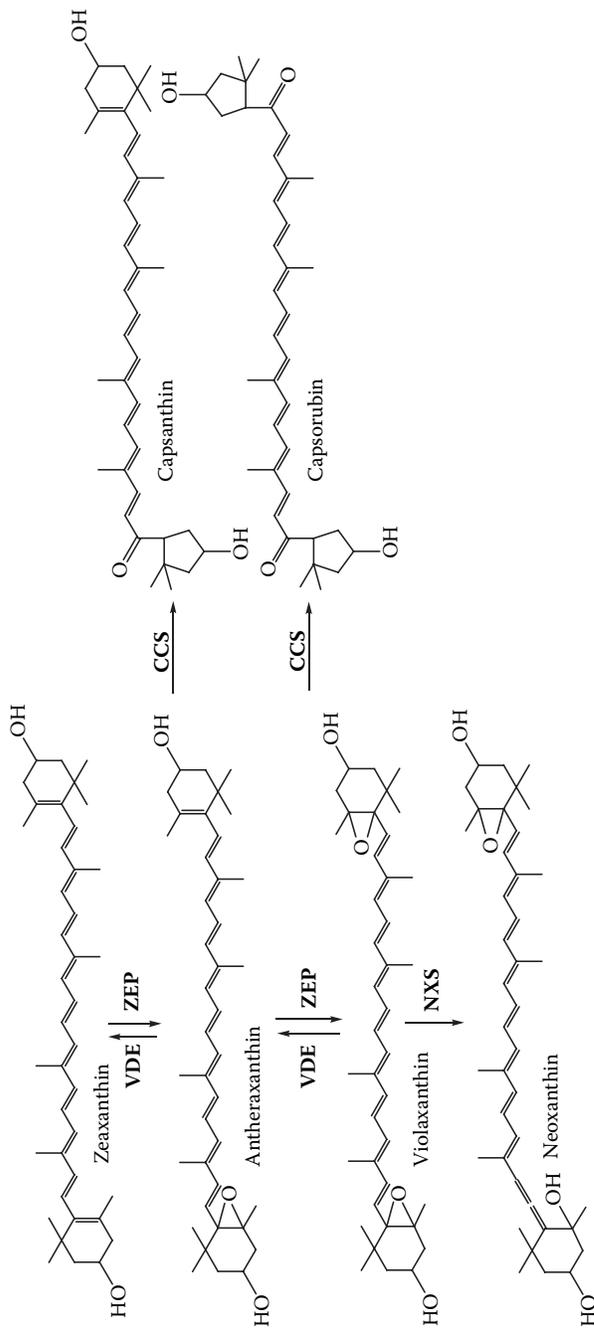
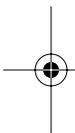


FIGURE 5.3.3B Oxidation to ketocarotenoids, capsorubin, and capsanthin. Enzyme abbreviations and enzyme activities are defined in Table 5.3.1.



of the  $\beta$ -ring,  $\epsilon$ -ring, or both rings of  $\alpha$ -carotene produces zeinoxanthin,  $\alpha$ -cryptoxanthin, and lutein, respectively (Figure 5.3.3A). Based on genetic studies of *Arabidopsis*, the  $\beta$ -ring hydroxylase was proposed to be a P450 enzyme in the CYP97 family, CYP97C, whereas the  $\beta$ -ring hydroxylases include the related P450 enzyme, CYP97A, and a nonheme di-iron monooxygenase, HYDB, homologous to the enzyme found in bacteria.<sup>169,170</sup>

Enzyme activities for the CYP97 enzymes from rice were directly demonstrated in *E. coli*; CYP97C was shown to be specific for  $\epsilon$ -rings and CYP97A was found to prefer primarily a  $\beta$ -ring substrate but also showed weak activity towards the  $\epsilon$ -ring substrate<sup>171</sup> as had been reported for the nonheme di-iron  $\beta$ -ring hydroxylases.<sup>172</sup> However, it is not entirely clear whether the two structurally distinct  $\beta$ -ring hydroxylases function on specific branches of the pathway in plants. For example, while the P450 CYP97A could hydroxylate  $\beta$ -carotene in *E. coli*, it may be that the enzyme is not in a biochemical context to do so in the plant.

Furthermore, it is unclear whether the structurally distinct  $\beta$ -ring hydroxylases can compensate for each other if activity of one is diminished. These are important issues to address in engineering strategies for  $\beta$ -carotene accumulation which include blocking of  $\beta$ -ring hydroxylase activity. Moreover, the presence of gene families further complicates strategic engineering of provitamin A accumulation. For example, *Arabidopsis* has two genes encoding the di-iron enzyme and one gene each for the P450 enzymes. In maize, numerous genes (and pseudogenes) code for the di-iron enzyme.<sup>16</sup>

A phylogenetic analysis of HYD proteins among monocots, daffodil and crocus, as well as dicots, suggests several independent duplications of *Hyd* genes during land plant evolution.<sup>173</sup> Importantly, accumulation of the crocetin apocarotenoid was shown to be primarily correlated with *Hyd* transcript levels.<sup>173</sup> Understanding of the complex interactions and overlapping actions of hydroxylase activities and multiple hydroxylase loci is essential for breeding strategies for high-level accumulation of  $\beta$ -carotene requiring blocking or selection of tissue-specific null mutants of hydroxylase activity in non-photosynthetic plant tissues.

### 5.3.2.7 EPOXIDATION OF XANTHOPHYLLS: BIOSYNTHESIS OF ANTERAXANTHIN AND VIOLAXANTHIN

The 3-hydroxyl  $\beta$ -rings of zeaxanthin are further oxygenated by the introduction of 5,6-epoxy moieties by zeaxanthin epoxidase (ZEP).<sup>174</sup> A mono-epoxidated intermediate, antheraxanthin is produced, followed by the di-epoxy xanthophyll, violaxanthin, as shown in Figure 5.3.3B.

Physiologically, violaxanthin is an important component of the xanthophyll cycle;<sup>175</sup> a high light stress-induced de-epoxidation of the violaxanthin pool to the more photoprotective zeaxanthin is mediated by violaxanthin de-epoxidase (VDE). Violaxanthin and neoxanthin, an enzymatically (NXS)-produced structural isomer, are the precursors for the abscisic acid (ABA) biosynthetic pathway (Figure 5.3.1, Pathway 4 and Figure 5.3.2). In non-photosynthetic tissues, namely: ripe bell peppers, antheraxanthin and violaxanthin are precursors to the red pigments, capsanthin and capsorubin, respectively (Figure 5.3.3B).

### 5.3.2.8 KETOCAROTENOIDS

#### 5.3.2.8.1 Ketolation to Capsanthin and Capsorubin

A lycopene cyclase-related enzyme, capsanthin/capsorubin synthase (CCS), converts the cyclohexane end rings of antheraxanthin or violaxanthin to cyclopentane rings to produce capsanthin or capsorubin, respectively.<sup>176</sup> The enzyme acts on the 3-hydroxy-5,6 epoxy group to produce the unusual  $\kappa$ -ring. Capsanthin is a mono-ketolated product; capsorubin is di-ketolated (Figure 5.3.3B). Among plants, these ketocarotenoids are limited to species of *Capsicum*. All plants make antheraxanthin and violaxanthin (they have ABA), so that transplantation of only one gene may allow accumulation of these red pigments in other plants. Engineering of tissue-specific expression is needed to avoid perturbations of normal physiology in photosynthetic tissues (zeaxanthin) and seeds (ABA).

#### 5.3.2.8.2 Astaxanthin

Astaxanthin provides wild salmon with their characteristic deep orange hue; this is a commercially valuable ketocarotenoid that has been chemically synthesized for feeding to farmed fish in order to produce optimal coloration desired for marketing. In algae and fungi, astaxanthin accumulates in response to stress. Astaxanthin is not naturally occurring in most higher plants. Whereas *Capsicum* ketocarotenoids are derived from zeaxanthin, the biosynthesis of the ketocarotenoid astaxanthin usually occurs by a different biosynthetic route that does not involve zeaxanthin.<sup>177</sup>

A single  $\beta$ -C-4 oxygenase introduces keto groups at the 4 and 4' positions of  $\beta$ -carotene rings, producing the intermediate echinenone followed by canthaxanthin. The enzyme modifies both end rings, analogous to HYDB and CYP97A. A carotene hydroxylase then introduces 3 and 3' hydroxyls to each ring. Astaxanthin biosynthesis in *Adonis* utilizes a different mechanism having evolved an enzyme with similarity to plant di-iron  $\beta$ -ring hydroxylases.<sup>178</sup> Introduction of astaxanthin biosynthesis has been accomplished in tobacco with a combination of a unicellular algal ketolase and endogenous hydroxylase.<sup>177</sup>

### 5.3.2.9 CAROTENOID CLEAVAGE AND APOCAROTENOIDS

Apocarotenoids are the derivatives of cleaved carotenoid chains (Figure 5.3.1, Pathway 4). Cleavage involving oxygen occurs at double bonds. These carotenoid cleavage products (CCPs) are often substrates for further enzymatic modification. While apocarotenoids with new functions such as intercellular signaling<sup>179</sup> have only recently been discovered, the origin of CCPs from carotenoids has long been suspected.

Well-known CCPs and their functions include (1)  $\beta$ -ionone, geraniol, and  $\beta$ -damascenone (fruit and flower flavor and fragrance), (2) ABA (dormancy, stress, and senescence phytohormone), (3) strigolactone (mycorrhizal fungal and parasitic weed growth stimulant),<sup>180-182</sup> (4) mycorradicin and blumenin (phytoalexin antifungals), and (5) trisporic acid (suspected pheromone).<sup>183</sup> Their genesis is now better defined by newly isolated gene functions that were recently reviewed.<sup>179,183,184</sup> They are produced by carotenoid cleavage dioxygenases (CCDs), otherwise referred to as

carotenases (Table 5.3.1).<sup>179</sup> Apocarotenoids are also produced in animals and include the retinoids: retinal (vitamin A), retinol (vision), and retinoic acid (morphogen).

The mammalian enzymes involved in vitamin A and apocarotenoid production cleave a variety of substrates (50 to 60 dietary carotenoids!) at central or eccentric double bonds and have been recently reviewed.<sup>185</sup> A mammalian CCD-homologous protein acts as a retinal isomerase,<sup>185</sup> but no plant CCD homologues reported have shown carotenoid isomerase activity. Here we will briefly describe the general properties of CCD enzymes and their role in plant physiology as it may relate to bioengineering of plant carotenoid and apocarotenoid accumulation. After the pertinent review, we will relate recent progress in molecular genetics of specific food pigments, bixin and saffron.

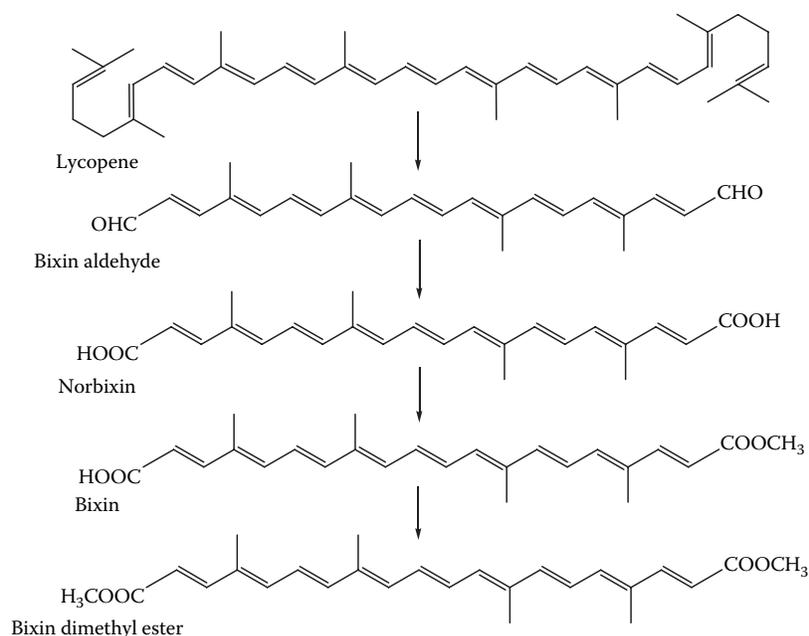
Plant apocarotenoids have a wide variety of structures and functions. As expected, there is a small gene family of CCDs with different cleavage sites and somewhat promiscuous substrate selection. Some CCDs are stereo-specific, for example, 9-*cis* epoxy-carotenoids are the substrates for NCEDs (9-*cis* epoxy dioxygenases) that produce the precursor of ABA biosynthesis, xanthoxin. Both linear carotenoids (lycopene) and cyclic carotenoids are substrates for cleavage at various double bonds including the central 15-15' and eccentric 5-6, 7-8, 9-10, 9'-10', and 11-12 bonds. Some CCDs cleave both linear and cyclic carotenoids and may cleave the same molecule twice, e.g., both 9-10 and 9'-10' positions.

The first CCD gene cloned was *viparous* 14 from maize, for which the recessive allele conditioned ABA deficiency.<sup>186</sup> Other CCDs among several plants have been cloned by homology and functionally demonstrated. Arabidopsis was found to have nine CCD-homologous genes, of which five are involved in ABA physiology, two in morphogenic signal molecule production, one that produces two  $\beta$ -ionones from  $\beta$ -carotene, and one, CCD1, is highly promiscuous, producing a large variety of products.<sup>183</sup> Importantly, CCDs not only condition accumulation of dialdehydes and ketones, but also affect carotenoid turnover, and thus, may negatively impact pigment accumulation in some tissues, as seen in white chrysanthemum petals: transgenic blockage of CCD expression revealed the underlying carotenoid biosynthesis for which accumulation was ordinarily prevented through turnover mediated by CCD activity.<sup>187</sup>

#### 5.3.2.9.1 Bixin (Annatto)

Bixin, or annatto, is a widely used food colorant, an apocarotenoid produced only by the neotropical plant, *Bixa orellana*. In an elegant study using standard molecular techniques and a bacterial complementation system, the genes involved in the biosynthesis of bixin were cloned and functionally characterized.<sup>188</sup> The pathway determined by the gene isolation is shown in Figure 5.3.4A and reviewed in the recent literature.<sup>183</sup>

The three genes that mediate the oxidative modification of lycopene to the carboxylic monomethyl ester apocarotenoid, bixin, were transferred to a variety of *E. coli* strains producing lycopene or other carotenoids to prove function and substrate range of the encoded gene products. The *B. orellana* lycopene cleavage dioxygenase (BoLCD) was specific for lycopene and did not cleave  $\beta$ -carotene or zeaxanthin. Thus, BoLCD did not seem to be substrate-promiscuous, as is, for example, CCD1 of Arabidopsis. Bixin methyl ester accumulated to moderate levels



**FIGURE 5.3.4A** Proposed biosynthetic pathway to bixin. Enzyme abbreviations and enzyme activities are defined in Table 5.3.1.

*Continued.*

in the heterologous host, demonstrating the function of the genes in the production of the pigment.<sup>188</sup>

Genes for the predicted bixin aldehyde dehydrogenase and carboxy methyltransferase, norbixin methyltransferase, which mediates the accumulation of the apocarotenoid dimethyl ester, were cloned from *B. orellana* by using an RT-PCR primer based on other plant sequences. PCR products were used to screen a developing seed cDNA library by hybridization. Isolation of genes from cDNA of *B. orellana* was based on homology to previously cloned carotenoid cleavage dioxygenases from *Arabidopsis* and maize.<sup>186,189,190</sup> Figure 5.3.4A shows the structures of intermediates and the accumulated bixin dimethyl ester. Bouvier et al. suggest that these three genes may be sufficient to genetically engineer the accumulation of bixin in tomato, which ordinarily accumulates high levels of lycopene.<sup>188</sup> Production of bixin in transgenic tomatoes or another crop may offer a cost-effective alternative to production of this food colorant in temperate zones.

### 5.3.2.9.2 Crocetin and Safranal

The aroma and red color of the spice saffron are partly due to the style-specific accumulation of carotenoid cleavage products produced by both enzymatic<sup>173,191,192</sup> and thermal degradation.<sup>193</sup> M. Giaccio reviewed the renewed interest in saffron as a colorant, spice, and nutraceutical.<sup>194</sup> Crocetin is a C<sub>20</sub> apocarotenoid derived from zeaxanthin (Figure 5.3.4B).<sup>191</sup>





UDP-GTase may be associated with membrane transporters and is a key component of pigment accumulation for the metabolic engineer to consider.

### 5.3.3 CAROTENOID BIOTECHNOLOGY

Conventional breeding often focuses on introgression of a value-added trait such as disease resistance, yield increase, or accumulation of valued metabolites. Often a new trait is brought into elite commercial germplasm by mating to an exotic germplasm. Crossing brings to the selected progeny the desired trait and many other undesirable traits or inadvertently co-selected traits. Further breeding cycles are then required to reconstitute a commercially viable variety with the introgressed trait. Biotechnology aimed at crop improvement is often focused on introgression of stably inherited traits, with minimum disruption of the pre-selected adaptive state.

Biotechnologies such as mutation breeding, marker-assisted selection (MAS), and genetic engineering are thought to be superior methods of crop improvement because they increase the efficiency and accuracy of pinpoint introgression of value-added traits. Furthermore, the addition of traits from other species, for example, from bacteria to plants, can transcend the possibilities of conventional breeding. Nevertheless, biotechnology is not a panacea and presents its own problems and challenges.

#### 5.3.3.1 HETEROLOGOUS COMPLEMENTATION IN *E. COLI*

Unicellular algal and bacterial genes were the first to be isolated and characterized and led to the isolation of most of the higher plant genes involved in carotenoid biosynthesis. Carotenogenic gene clusters from bacteria and algae<sup>115,147,196–198</sup> contributed immensely to the elucidation of the carotenoid pathway.

Homology to algal genes and complementation in bacterial systems led to isolation and characterization of higher plant genes involved in carotenoid biosynthesis from a variety of model and crop species including *Arabidopsis*,<sup>151,156,172,199</sup> bell pepper,<sup>200</sup> maize,<sup>16,109,128,145,201,202</sup> rice,<sup>128,199,203</sup> soybean,<sup>133</sup> tomato,<sup>125,157,204</sup> and other plants.<sup>93,125,134,157,201,204–208</sup> Algal genes were essential to the isolation of higher plant genes, because the homology between bacterial genes and plant genes was generally too low to allow cloning of plant genes using hybridization-based approaches.

Transplantation of the genes to an *E. coli* expression platform allowed facile, stepwise addition of gene functions coupled with metabolite profiling and complementation of genes from other sources such as higher plants. For example, the function of a putative plant gene could be tested in the presence of a bacterial carotenogenic gene cluster which was missing the suspected gene function followed by HPLC analysis of metabolite accumulation. Whole cDNA libraries were screened visually for gene functions directly or indirectly affecting carotenoid accumulation, using a procedure called color complementation screening. Examples of using the heterologous system for isolation coupled with functional complementation are included by reference.<sup>106,109,124,128,172,199,202,203,209,210</sup> The jump from algal genes to plant genes and color complementation systems exerted a dramatic impact on the pace of progress in higher plant gene cloning and functional characterization.

Complementation systems were also used to examine the consecutive actions of plant genes. Determination of the function of more than one plant gene acting in concert was especially instructive when examining the effects of geometric isomer states of intermediates on the progression through the desaturation series using PDS and ZDS from maize<sup>145</sup> and Arabidopsis.<sup>151</sup> The paired desaturases were shown to mediate a poly-*cis* isomer pathway in *E. coli*, as discussed in Section 5.3.2.4.

The stereo-chemical behavior of PDS and ZDS from the phylogenetically disparate monocots and dicots as demonstrated with recombinant enzymes in *E. coli*, indicated the wide natural distribution of the poly-Z pathway among plants. The intermediates accumulating in the heterologous system and in particular plant loss-of-function mutants such as *tangerine* of tomato,<sup>152</sup> *carotenoid and chloroplast regulation 2 (ccr2)* of Arabidopsis,<sup>136</sup> and of algal mutants<sup>211–213</sup> allowed the possibility of isomerases that could regulate progression through the stereo-specific enzymes in the pathway. Subsequently, an isomerase was cloned by linkage analysis (map-based cloning) of the *tangerine* locus from tomato<sup>214</sup> and from the chemically induced mutant *ccr2* from Arabidopsis.<sup>136</sup> The algal isomerase was also quickly identified.<sup>160,161</sup>

Following the lead from bacterial and algal systems, the combination of classical genetics and biochemical analyses, map-based cloning, heterologous expression, and morphological analyses in plants demonstrated that an isomerase was involved in the desaturation series (reviewed by Giuliano<sup>163</sup> and Eckardt<sup>162</sup>), and has biochemical and morphological roles in development of carotenoid accumulation and chloroplasts,<sup>136</sup> thus ending a 60-year-old enigma of *cis*-carotenoid metabolic intermediates in plants. Recently, *in vitro* substrate feeding experiments with the recombinant tomato isomerase extract from *E. coli*<sup>143</sup> demonstrated specific substrate specificity of CRTISO,<sup>145</sup> but left open the possibility for an additional isomerase acting on 15-15'-*cis* phytoene, the recently discovered Z-ISO.<sup>266</sup>

### 5.3.3.2 GENETIC ENGINEERING

Proof-of-concept genetic engineering studies of plants with single or multiple transgenes aimed at the biotechnological improvement of food carotenoids have recently been cited in reviews.<sup>12,18,215</sup> Transgenesis into rice to create Golden Rice has been comprehensively reviewed.<sup>59</sup> Despite concerns about the effectiveness of these transgenic crops as nutraceuticals,<sup>59,216</sup> much progress has been made and several products may soon be released.

#### 5.3.3.2.1 Bacterial Genes

The bacterial genes *CrtB* and *CrtI*, coding functions equivalent to plant phytoene synthase (PSY) and phytoene desaturase (PDS/ZDS/CRTISO), respectively, have been engineered into several plants with mixed and sometimes unexpected but informative results. The native bacterial genes lack a chloroplast transit peptide and a plant promoter, so these eukaryotic regulatory and targeting elements were engineered into the transgene at the DNA level. For example, the small subunit (SSU) of RuBisCo (Calvin cycle enzyme) and CaMV 35S promoter (virus) have frequently

been used for targeting and strong constitutive expression. Tissue-specific promoters for seeds and fruits have also been used and are discussed specifically in this section.

PSY was shown to be rate-controlling for flux (discussed in Section 5.3.2.3) into carotenenes in a number of systems. Hence, *CrtB* was introduced into plants under the control of several different regulatory elements. Since tomatoes are the major dietary sources of lycopene and  $\beta$ -carotene in some countries, tomatoes have received the most attention as targets of engineering.<sup>132,174,217–220</sup> In 1995, Fray et al. reported the first use of over-expression under the control of the CaMV 35S promoter of tomato *Psy1* in tomatoes.<sup>130</sup> The results were not good and led to the later use of *CrtB* instead.<sup>122</sup> Specifically, plants over-expressing the fruit-specific tomato PSY1 in a constitutive manner were pleiotropically affected in a number of ways: (1) dwarfism due to depletion of the GGPP precursor pool shared with the gibberellic acid growth hormone, (2) hyperaccumulation of pigment in shoots and roots leading to intense coloration and photosensitivity, (3) increase in ABA levels due to increased carotenoids, and (4) premature accumulation and little to no accumulation of carotenoids in fruits that might be attributed to gene silencing due to the use of the endogenous tomato gene.<sup>12</sup>

*CrtB*, which has low-level nucleotide sequence homology to *Psy1*, was inserted under the control of the ripening, fruit-specific polygalacturonase promoter (PG). The results were good. *CrtB* was expressed in a fruit-specific manner and targeted to the plastid and resulted in a two- to three-fold increase in carotenoids including lycopene and  $\beta$ -carotene.<sup>122</sup> *CrtB* over-expression has also been effective in canola, carrots, and potatoes.

The case of canola is extraordinary because of the very high level accumulations (50-fold) of leaf-type carotenoids in seeds when the gene was introduced under the seed-specific promoter, napin.<sup>123</sup> The exalbuminous seeds of canola differ from those of genetically engineered rice cereal grains in that they have chloroplasts, which may explain the capacity for hyperaccumulation of carotenoids.

The food technologist may be especially interested in the fate of the carotenoids in the seed oil. Like red palm oil, the resulting carotenoid-pigmented canola oil may be more stable due to the antioxidant properties of carotenoids and may be more attractive to consumers. Alternatively, for food security concerns, transgenic soybean or canola oils and seed meals that are genetically modified for more efficient bio-diesel production may be bio-safety marked with lipid-soluble carotenoids and water-soluble anthocyanins, respectively. Potatoes are excellent potential sources of dietary carotenoids, and over-expression of *CrtB* in tubers led to the accumulation of  $\beta$ -carotene.<sup>221</sup> Potatoes normally have low levels of leaf-type carotenoids,<sup>222</sup> like canola cotyledons.

Over-expression of bacterial phytoene synthase led to only modest increases in pigment accumulation (except in the case of chloroplast-containing tissues). Attention turned to *CrtI*, one gene that might control flux through the entire four desaturation steps from phytoene to lycopene (discussed in Section 5.3.2.4). Only a modest increase in carotenoid content in tomatoes and a variety of changes in carotenoid composition including more  $\beta$ -carotene, accompanied by an overall decrease in total carotenoid content (no lycopene increase), resulted when *CrtI* was over-expressed under control of CaMV 35S.<sup>223</sup> Apparently, the bacterial desaturase

has pleiotropic effects on the pathway and a putative induction effect on LCYB without affecting compositions of geometric isomers.<sup>12,220</sup>

Manipulation of carotenogenesis with bacterial genes demonstrated the suspected ability of bacterial gene products to function in the plastid environment, the need for specific promoters, the advantage of heterologous genes in avoiding transcriptional silencing, and uncertainties surrounding communication and homeostasis within the pathway. While the bacterial functions worked, it is unclear whether they cause problems because they do not interact normally with the plastidial carotenogenic metabolon.

#### 5.3.3.2.2 Transplanting Plant Genes

Plant phytoene synthase (*Psy*) has been used in a variety of transgenics. As noted above, *Psy1* over-expression under a strong constitutive promoter caused a decrease in carotenoid accumulation, probably due to transcription silencing.<sup>130</sup> Similarly, over-expression of the gene sequence backward (antisense) also silenced activity.<sup>224</sup> In another approach to over-expression of tomato *Psy1* in fruits, a synthetic alternative in which the third position of each codon was changed in order to avoid transcriptional silencing was successful in conditioning an increase in carotenoid accumulation.

In Golden Rice, over-expression of daffodil *Psy*<sup>225</sup> and a variety of other plant *Psy* genes including the endogenous rice gene resulted in a wide range of increased accumulation of xanthophylls.<sup>226</sup> Because there are duplicate factors that vary in their evolutionary relationships and tissue-specific expression patterns (discussed in Section 5.3.2.3), it is notable that the maize and rice *Psy* genes in Golden Rice 2 conditioned accumulation of higher carotene accumulation compared to *Psy* genes from other plant families.

In rice endosperm that accumulated phytoene by virtue of daffodil *Psy* transgenesis, Burkhardt et al. reported the failure of daffodil *Pds* over-expression in rice endosperm to condition accumulation of  $\zeta$ -carotene, even though an increase in PDS antigen was detected.<sup>225</sup>

Success with transgenic expression of *Pds* or *Zds* or both has not been reported. Also not known is whether CRTISO and Z-ISO, the companion isomerases to the desaturations, will influence accumulation of intermediates or products.

The availability of precursor IPP may ultimately be most influential over accumulation of carotenoid metabolites.<sup>227</sup> While over-expression of DXS and DXR in color complementation systems leads to hyperaccumulation of carotenoids (discussed in Section 5.3.3.3), over-expression of plant *Dxs* genes has not always been effective. Over-expression of DXS resulted in increased carotenoid accumulation in transgenic tomato<sup>99</sup> and Arabidopsis,<sup>228</sup> but over-expression of daffodil DXS in rice endosperm did not increase pigment accumulation.<sup>59</sup>

Over-expression and anti-sense constructs of LCYB have been tested in rice and tomato. In Golden Rice, daffodil LCYB was over-expressed but found to be unnecessary for accumulation of carotenes. Only PSY and CRTI are needed to accumulate carotenes and xanthophylls in the endosperm.

Apparently endogenous LCYB (and HYDB) is constitutively expressed and was not induced by the presence of CRTI,<sup>229</sup> a not surprising fact since endogenous

expression of the pathway genes in rice endosperm had been known for some time.<sup>230,231</sup> In tomatoes that accumulate high levels of lycopene, Arabidopsis LCYB over-expression with a fruit-specific *Pds* promoter resulted in increased accumulation (five-fold) of  $\beta$ -carotene, while antisense over-expression of an antisense tomato LCYB under a *Pds* promoter caused a small increase in lycopene only. In both cases, leaf carotenoid content was not affected. Others had similar results with the second member of the LCYB gene family in tomato.<sup>232</sup>

Xanthophylls do not normally occur in tomato fruits but have particular health benefits. Tomato fruits are exceptional storage organs for carotenoids and thus are natural targets for nutritional enhancement. Transgenic expression of the plant or bacterial HYDB alone did not result in accumulation of zeaxanthin, but co-transformation of Arabidopsis LYCB together with Capsicum HYDB did.<sup>217,218</sup> Also in potatoes, Arabidopsis ZEP was over-expressed in both sense and antisense orientations in tubers under control of the granule-bound starch synthase (GBSS) promoter, resulting in silencing of the conversion of zeaxanthin to antheraxanthin and violaxanthin.<sup>233</sup> In various situations, zeaxanthin or antheraxanthin accumulated. Also, total carotenoid content increased, reminiscent of pathway up-regulation in tomato fruits perturbed by the addition of *CrtI*.

#### 5.3.3.2.3 Mixed Plant and Bacterial Genes

Some multigene transgenic plants have a mixture of added plant and bacterial genes. Golden Rice is a famous example. Golden Rice 1 contains the transgenes daffodil *Psy*, bacterial *CrtI*, and daffodil *LcyB*. The addition of daffodil PDS failed to confer colored carotenoids in rice endosperm.<sup>225</sup> Unbeknownst at the time, the use of the plant two-step desaturases, PDS and ZDS, might also require the addition of companion isomerases. Based on the substrate specificity of CRTISO for the products of ZDS,<sup>143</sup> it is unlikely that a lack of CRTISO affects accumulation of the products of PDS, phytofluene and  $\zeta$ -carotene. In any event, the lack of accumulation of products of daffodil PDS in rice endosperm precipitated the use of *CrtI*.<sup>234</sup>

Fortuitously, the bacterial gene product, CRTI, produces all-*trans* carotenoids and satisfies the stereo-chemical specificity of LCYB for all-*trans* substrates while also catalyzing the four desaturation steps from phytoene to lycopene. Nevertheless, over-expression of *CrtI* has been shown to have only a modest effect (two- to four-fold increases in tomatoes and carrots) in increasing flux through the pathway and some unexpected pleiotropic influences on activities upstream and downstream of the desaturations (reviewed by Fraser and Bramley<sup>12</sup> and Giuliano<sup>220</sup>).

Recently reported experiments indicate that modulation of CRTI activity with various promoters does not appreciably affect carotenoid accumulation in rice endosperm.<sup>59</sup> Transgenic manipulation of PDS/ZDS/CRTISO, and Z-ISO when available, for crop improvement has yet to be reported, but nevertheless may present advantages over the transgenic bacterial desaturase. For example, use of bacterial genes may have a disadvantage in deregulatory issues. The transgenesis of bacterial genes to edible plants requires higher level regulatory approval (not required for genes from one edible plant to another edible plant in the United States). Transgene products from non-crop sources, such as bacteria or daffodils, are less appealing to



the public than the use of plant genes, whose products have a history of use in food and perhaps require fewer deregulatory costs and less effort.<sup>59</sup>

There is already some indication in genetic manipulations of carotenogenesis that transgenes from closely related species perform better than do genes encoding evolutionarily distant isozymes. In Syngenta's Golden Rice 2 which is thought to be a practical nutraceutical,<sup>25</sup> the use of maize PSY over-expression in rice endosperm led to the highest levels of carotenoid accumulation among the other PSYs tested, including Arabidopsis, carrot, daffodil, bell pepper, rice, and tomato.<sup>58</sup> This is in contrast to over-expression of DXS and CRTI, which had little effect on accumulation.

Now that the flux-limiting step (PSY) has been determined, partially optimized, and opened, it is compelling to give new attention to flux through the desaturations, and for reasons cited above, even more compelling to consider PDS/ZDS/isomerases coded by rice or maize genes as an alternative to *CrtI*. Maize genes may be preferable in rice because they are public,<sup>118,128,145,201</sup> likely to have enough nucleotide dissimilarity to evade transcriptional silencing (discussed in Section 5.3.2.2), and may interact more appropriately with a carotenogenic metabolon.

#### 5.3.3.2.4 Antisense Approaches and RNAi

Disruption of gene function by transcript-level attenuation is often used to assess function and can also be used to effect desired changes in accumulation of pigments. Examples of experimental gene suppression were already discussed in relation to applying VIGS technology to investigate MEP pathway functions (Section 5.3.2.2) and in manipulation of carotenogenic enzymes in tomatoes.

In a very recent study in potatoes, inhibition of LCYE accumulation was accomplished by an antisense *LcyE* driven by the patatin promoter and allowed rechanneling of lycopene toward the  $\beta$ -carotene branch of the pathway to produce up to 14-fold increased levels of  $\beta$ -carotene as well as up to 2.5-fold increased total carotenoids.<sup>235</sup> RNAi and TILLING for manipulation of carotenogenesis have yet to be reported, but these new techniques for suppression of function and generation and selection of allelic diversity are likely to impact future research and production of varieties with enhanced pigment accumulation.

#### 5.3.3.3 QTL AND ASSOCIATION GENETICS

Association of pigment accumulation with DNA features can inform systematics and applied breeding in crop plants. For carotenoid content among crop plants, maize and tomato are best studied by this technique. Because there is interest in accumulation of various intermediate products in the pathway as well as end products, QTLs were developed for genetic determinates that are not independent of each other.<sup>236</sup> Some QTLs affect multiple pigment contents.

In maize, many phenotypic mutants have been associated with cloned genes by a combination of HPLC analysis of specific intermediate metabolite accumulation, RT-PCR and immunolocalization of candidate genes, and recombinant inbred mapping of candidate cDNAs.<sup>16</sup> *Psy1* was cloned by transposon tagging<sup>237</sup> and later shown to be functional in the color complementation system and to be the specific

isoform (not *Psy2*) expressed in maize endosperm.<sup>118,128,137</sup> *Psy* was associated with the *Y1* mutant by a transposable element-induced instability of pigment accumulation (somaclonal sectors)<sup>238,239</sup> and genetic and recombinant inbred linkage mapping.<sup>237</sup> *Pds* and *Zds*, which are single copy genes, were also associated with the mutants, *vp5* and *vp9*, by a series of similar studies.<sup>118,145,201,202,238,240–242</sup>

QTL analysis of the intermediates in the pathway (such as  $\beta$ -cryptoxanthin, see Figure 5.3.2) and of total accumulated carotenoids associated the variation in content with *Y1* and *Vp9* but not with *Vp5*. Candidate genes *Psy* and *Zds* but not *Pds* were thus associated with the variation in specific and total pigment contents. These influential genes and associated markers can now be used to further select allelic variants by association genetics or TILLING and produce selection tools (MAS) to use in a conventional breeding strategy that may lead to targeted crop improvement. One such target is an increase in  $\beta$ -carotene content for sub-Saharan maize staples.<sup>236</sup>

Most of the DNA variation that affects pigment accumulation is thought to be associated with mutations in the *cis*-acting regulatory elements, most often upstream of the structural coding regions.<sup>236</sup> LD mapping studies conducted on a wide collection of germplasm associated changes in the 5' *cis*-acting regulatory sequences of *Psy1* with the co-evolution of yellow endosperm during American maize domestication.<sup>75,243</sup> Specifically, a transposable element in the promoter of *Psy1* seems to have led to tissue-specific gain of function, associated with novel accumulation of carotenoids in kernels, compared to the white endosperm progenitor. Again, *Psy2* was not associated with pigment accumulation in the endosperm,<sup>243</sup> as was predicted and confirmed by classical molecular genetic studies.<sup>106,118,128</sup>

Thorup et al. exploited the microsynteny of bell pepper and tomato and cloned candidate genes from each to determine correspondence of QTL.<sup>71</sup> A comparative approach across species places consensus QTLs, candidate genes, and biochemistry in a phyletic perspective avoiding uncertainties associated with environmental and ontogenic variation in traits.

QTLs were also detected in tomato introgression lines (domestic tomatoes with single, defined chromosome regions selected from the progeny of a cross with a wild, non-pigmented variety) that correspond to candidate loci<sup>244</sup> for which genes and mutants have been previously characterized. Detected QTLs included the *r* locus (*Psy*), the *Del* locus (*LcyE*), and the *B* locus. Similar to the tissue-specific expression of maize *Y1*, the *B* locus codes a fruit-specific lycopene cyclase associated with higher levels of  $\beta$ -carotene.<sup>12</sup>

The stability of QTL during applied evolution of tomatoes has also been assessed for organoleptic qualities<sup>245</sup> and transcriptomics, and select metabolomics have now been applied to fruit ripening.<sup>246</sup> Such phyletically broad and pan-cellular studies are at the forefront of development of an integrative approach to understanding pigment accumulation in a broader sense that transcends poke-and-hope genetic engineering.

#### 5.3.3.4 GENERATION OF VARIATION

Experiments in directed breeding have been carried out in bacteria and are proving grounds for metabolic engineering of pigment accumulations in plants. Experi-

ments with gene shuffling have shown that novel carotenoids can be produced by mixing genes from gene libraries. C. Schmidt-Dannert et al. demonstrated gene shuffling for carotenoid functions and reviewed<sup>33,77,247,248</sup> similar efforts such as those of Wang et al.<sup>249</sup>

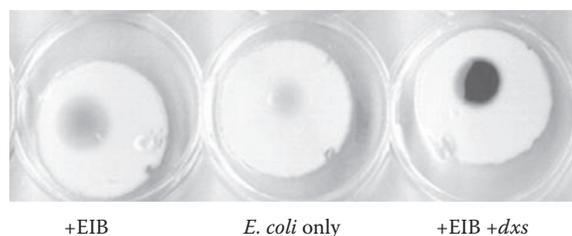
Because of the ease of screening for color variants and the ability to recursively add new functions to a color complementation system, the carotenogenic model served as a testing ground for shuffling concepts. By first shuffling two *Erwinia* desaturase genes and then adding a library of shuffled lycopene cyclase genes, a directed evolution scheme produced tetrahydrolycopene and then torulene in *E. coli*.<sup>77</sup> Accumulation of torulene was a newly evolved function among the progenitor gene set. The addition of further downstream functions such as hydroxylase, ketolase, or glycosylase resulted also in remodeled torulenes.<sup>250</sup> Accumulation of the novel products was then improved by more typical microbial production optimization.<sup>248,251</sup> Further progress in directed evolution of carotenogenic genes has also been reported.<sup>107,252</sup> Thus, the color complementation system has also been used effectively for testing concepts in metabolic engineering.

### 5.3.3.5 METABOLIC ENGINEERING

Transgenic *E. coli* accumulate comparatively low levels of carotenoids<sup>253,254</sup> compared to microbial algae, yeasts, and bacteria. Many efforts<sup>36,37,250</sup> have focused on increasing accumulation by manipulation of factors affecting metabolic flux and metabolite accumulation (listed and discussed in Sections 5.3.1.1 and 5.3.1.3 A) and have been reviewed.<sup>118,254</sup> In bacterial systems, approaches to control can be categorized as either infrastructural (plasmids, enzymes, strains) or ultrastructural (media and feeding, environment, precursor pools, substrate flux).

Consultation of the cited literature will supply many examples of metabolic controls in microbial carotenogenesis. Influential infrastructural factors include: genetic background and strain selection, substrate feeding and optimization of growth and/or media conditions, stabilization of mRNA by alteration of gene structure, decrease of general metabolic burden by use of low copy number plasmids, use of inducible gene expression and optimization of the degree and timing of gene induction. Ultrastructural controls include: manipulation of metabolic flux by over-expression of rate-controlling enzymes, precursor pool enhancement, precursor pool balancing, removal of feedback inhibition by modification of gene and enzyme structure and sequestration of products. Some key examples are discussed in this section as a preface to prospects for higher plant metabolic engineering.

Careful empirical selection of the expression platform for carotenogenesis has included selection of the best strains for stability and degree of accumulation<sup>254,255</sup> and the selection of compatible drug-resistance combinations and low copy number polycistronic plasmids to enhance product accumulation by decrease of metabolic burden.<sup>118,145</sup> Matthews and Wurtzel discussed culture and induction conditions<sup>145,261</sup> that have been explored in most studies. Most efforts to engineer carotenoid biosynthesis in *E. coli* focused on the genes and enzymes of the pathway and had a modest effect on improved accumulation. For example, substitution and over-expression of a GGPPS that uses IPP directly (discussed in



**FIGURE 5.3.5** Enhancement of lycopene accumulation in *E. coli* by over-expression of DXS. Lycopene accumulation (left) is enhanced (right) when *E. coli* cells carrying a carotenoid pathway gene cassette (+EIB) are further transformed with a *dxs* gene on a multicopy plasmid (+EIB +*dxs*). Lycopene hyperaccumulation was demonstrated by Matthews and Wurtzel.<sup>261</sup>

Section 5.3.2.3) combined with over-expression of IPPI resulted in enhanced astaxanthin accumulation to 1.4 mg/g dry cell weight (DCW).<sup>249</sup> Further increases to 45 mg/g DCW were obtained by random mutagenesis of GGPPS,<sup>258</sup> perhaps by altering enzyme response to substrate-level feedback inhibition.

Tunable promoters that control the expression of key carotenogenic enzymes by sensing excessive flux through glycolysis were used to test concepts of dynamic flux control using bacterial carotenogenesis.<sup>258</sup> The balance of glycolytic precursors (pyruvate and G3P, see Figure 5.3.1) was modulated by over-expression of phosphoenolpyruvate synthase (PPS) or inactivation of pyruvate kinase. In conjunction with tunable promoters for PPS and IPPI expression, carotenoids accumulated five-fold over controls.<sup>259</sup>

The stability of phytoene desaturase and lycopene cyclase transcripts also influenced accumulation of carotenoids.<sup>259</sup> Efforts in directed evolution of carotenogenic enzymes have also continued. Alternate approaches using systematic and combinatorial gene knockout targets have allowed for enhancement of carotenoid production in the absence of *a priori* assumptions of regulatory mechanisms.<sup>106,260</sup>

Another approach to metabolic control of carotenoid accumulation was the modulation of the rate-controlling activities, DXS and DXR, of the MEP pathway (discussed in Section 5.3.2.2). Early experiments with the addition of DXS<sup>261</sup> and then DXR<sup>264</sup> showed that the precursor pool (DMAPP and IPP) was limiting for accumulation of lycopene and zeaxanthin in various color complementation systems. For example, over-expression of only DXS coded on a high copy number plasmid in the presence of a carotenogenic gene cluster on a low copy number plasmid resulted in enhanced lycopene accumulation to 1.3 mg/g DCW.<sup>63</sup> The addition of a DXS transgene resulted in a striking change in pigment accumulation of colonies as shown in Figure 5.3.5.

Over-expression of multiple enzymes in plants is also possible, especially as transformation technology improves. Multiple carotenogenic genes such as *Dxs*, *Dxr*, *Hdr*, *Ippi*, *Psy*, etc. may now be coordinately expressed in a plastidic polycistron.<sup>16,173</sup> Alternatively, genetic hybridization may be used to empirically effect a “balance” of gene expression and a metabolic optimum by employing either naturally diverse germplasm or primary transgenics that exhibit a wide range of position effect variation in expression. Since major control of accumulation is effected at the level



of gene transcription for plant carotenogenesis,<sup>16,257</sup> such transcriptional balancing may be a good semi-rational approach.

Several basic concepts in metabolic engineering of microbial carotenogenesis have also been explored in plants. For example, over-expression of enzymes shown to be rate-controlling in bacterial dissections<sup>261</sup> have proven to release flux in plants. As a case in point, over-expression of CrtI or PSY led to varied and sometimes dramatically positive results (discussed in Sections 5.3.3.2 and 5.3.2). Also, modulation of precursor pools, as demonstrated in bacteria,<sup>99,265</sup> occurred during over-expression of DXS in transgenic tomato<sup>228</sup> and *Arabidopsis*,<sup>59</sup> while over-expression of daffodil DXS in rice endosperm did not increase pigment accumulation.<sup>122</sup> Metabolic flux analysis in tomatoes<sup>33</sup> demonstrated that PSY is rate-controlling and confirmed empirical results of poke-and-hope genetic engineering.

One must remember that quantitative evaluations of pigment accumulation in transgenic plants are always complicated by variable position effects for individual events combined with genetic, developmental, and environmental noise; therefore more studies and statistical analysis of many events are needed for true consensus development. Also, because of difficulties of plant transformation, especially the genotype-limited applicability of optimized transformation protocols, wide-ranging empirical exploration of pigment accumulation platforms that vary in infrastructure such as genetic background, expression vector, and promoter strength is not currently tractable in plants.

The accumulation of lycopene seen in Figure 5.3.5 probably represents the maximum holding capacity of lipophilic compartments of bacterial cells. Further accumulation may require selection of alternative hosts such as yeasts or photosynthetic bacteria, or the understanding and engineering of novel sequestory structures.<sup>187</sup> In plants, product transport and storage may well limit accumulation at some point, as might the activities of degradation enzymes.<sup>195</sup> Consideration of product accumulation systems like the vacuolar deposition of crocetin glycosides<sup>20</sup> described in Section 5.3.2.8 and other membrane transporters becomes crucial.<sup>72,74</sup> True application of combined transgenesis, metabolomics, and MCA has yet to be reported for plant carotenogenesis.

#### 5.3.4 CURRENT STRATEGIES AND FUTURE PROSPECTS

Genomic and molecular tools have made great impacts on plant biotechnology and offer potential for manipulation of carotenoids as natural colorants and also for applications in human and animal health. While microbial and other non-plant systems have been successfully used, plant modification eliminates need for expensive bioreactors and offers economically feasible opportunities for less developed nations for production of nutraceuticals and other chemical products.

Plant use is less biotechnologically advanced and fundamentally more complex. The first generation of plant metabolic engineering met with mixed success and produced unanticipated results — problems that are not necessarily restricted to manipulation of carotenogenesis. The reason is that predictive metabolic engineering relies on the establishment of both needed tools and an information infrastructure



that are only just developing. The following are some of the essential tools and information required for predictive engineering of plant carotenogenesis.

1. *Characterization of gene families and identification of transcription factors to drive coordinate pathway expression:* Unlike microorganisms, agronomically important plants have gene families coding biosynthetic enzymes for which specific gene family member roles are poorly understood. These roles impact carotenoid involvement in both primary and secondary metabolism, including roles as precursors to apocarotenoids whose functions and regulation are even less understood. Although it is likely that individual members have specific functional modularity in different tissues or in response to different signals, they may also have overlapping functions. Manipulation of a gene family member to alter carotenoids in one tissue could potentially have a negative impact on carotenogenesis in other tissues. Therefore, an essential goal is a better understanding of the roles of gene family members in tissues targeted for manipulation as well as throughout the plant. Characterization of gene family members will potentially lead to identification of transcription factors that may or may not be involved in coordinate pathway induction, a valuable tool for global pathway enzyme manipulation.

2. *Understanding timing of gene expression:* Little is known about the critical timing for the expression of enzymes produced by either endogenous genes or foreign transgenes. Since carotenoid biosynthesis is nuclear encoded but plastid localized, carotenogenesis is likely intertwined with plastid development. Therefore, transgene expression to control carotenogenesis in endosperm, for example, is dependent on developmental timing of gene expression; use of a generic, albeit tissue-specific, promoter in the absence of such temporal consideration may be reflected by less than optimal results.

3. *Understanding mechanisms controlling metabolon localization in plastids of different membrane architectures:* Little is known about metabolon structure, assembly, and membrane targeting. The carotenoid biosynthetic pathway exists on plastid membranes. However, plastids have different membrane architectures and therefore tissue- and plastid-specific differences in membrane targeting of the biosynthetic metabolon can be expected. Localization in chloroplasts that harbor both thylakoid and envelope membranes differs from the envelope membranes in endosperm amyloplasts. In fact, localization on both thylakoid and envelope membranes implies that the carotenoid pathway is really not a single pathway, but a duplicated pathway that may very well have membrane-specific roles with regard to functions in primary and secondary metabolism.

Little is known of how the biosynthetic metabolon is assembled, what mechanisms control the membrane-specific targeting, and how the conversions to apocarotenoids occur. Yet the current approach to drive import of bacterial or plant genes is to use transit sequences of a stromal protein that may limit the effectiveness of the transgene. In addition, for specific applications of controlling carotenoid composition, we need to better understand the interactions of the various enzymes,



especially the structurally distinct hydroxylases that may have overlapping functions. Lastly, little is understood of substrate import into plastids and how this impacts carotenoid accumulation in time and space.

*4. Test systems to establish rate-controlling steps:* One cannot predict engineering results in genetically diverse populations or accessions based on testing a transgenic in a single model variety. Generally, a few amenable lines are routinely used for plant transformation in a given species. While such lines are important for the introduction of a new trait, they have limited value in predicting metabolic performances in diverse populations that are genetically and thereby chemically dissimilar.

How can a metabolic engineer predict when to maximize gene expression when carotenoid accumulation is linked with temporal control of gene expression during tissue development? What is the range in genotypic diversity with regard to plant chemistry? An alternative is to investigate gene expression in genetically diverse populations and carefully correlate gene expression data with plant chemistry. For example, the Wurtzel laboratory has been conducting gene transcript profiling in diverse maize germplasm and identified not only which gene family members are critical for impacting endosperm carotenogenesis, but also when during endosperm development expression is critical (Wurtzel et al., unpublished data). From these data, identification has been made of gene targets as well as developmental time frames needed for identifying better promoter and/or gene/allele choices leading to precise expression of rate-controlling enzymes.

Analysis in diverse lines can facilitate identification of useful alleles that control expression of enzymes upstream of the carotenoid pathway, a feature that would not be evident from conventional end-product screening of breeding lines. Moreover, this characterization sets the stage for marker-assisted selection of superior endogenous alleles and facilitates selection of introduced transgenes that may be necessary to supplement the genotypic contribution required for a particular plant chemical outcome.

*5. Development of multidimension pathway databases:* Current database tools do not encompass the true complexities of plants. In this regard, multidimensional databases need to be developed to integrate allelic variation with temporal, developmental, tissue-specific, and biotic and abiotic influences on pathway flux and pigment accumulation.

*6. Reliable phenotyping:* In the post-genomic era, it has become easier to measure genetic variation in a high throughput manner than to measure chemical trait data and morphological characteristics. Association studies such as QTL analysis rely on extensive genotyping of populations but also require several-fold higher levels of phenotyping. Many individuals in environmentally replicated mapping families must be phenotyped for many chemical and morphological traits in a reproducible fashion over several seasons of plant growth. Similarly, MCA is based on comparison of metabolite profiles for hundreds of metabolites.

Data production, assurance and validation are significant challenges requiring not only advanced analytical instruments, for example, for molecule separation and



identification or for high throughput videometrics, but also data handling and information flow, audit tracking, and statistical and computational advancements.

*7. Predictable transformation technologies:* Such needed technologies include higher-efficiency methods of gene transformation and standardized as well as more predictable results that might be obtained by targeted homologous recombination methods.

Predictable manipulation of carotenogenesis requires answers to many open questions and development of new biological, analytical, and computational tools. These are shared goals for rational, predictive metabolic engineering of secondary metabolite accumulation in plants. Advances in understanding control of carotenogenesis can be further applied to manipulation of interfacing native pathways and integration of non-native pathways. By application of comparative genomics, information gained by study and manipulation of one plant species can be further used to build resources and knowledge needed to modify and enhance related species. Multidisciplinary collaborations among fields including genomics and molecular biology, chemistry, biochemistry, ecology, biophysics, informatics, and computational science will lead to the advances necessary for making predictive metabolic engineering a reality.

## ACKNOWLEDGEMENTS

We would like to thank current members of ETW's laboratory, C. Murillo, R. Quinlan, and R. Vallabhaneni, for preparing pathway figures and also F. Li and former members for thoughtful discussions and contributions to advancing ongoing research on endosperm carotenogenesis in cereal crops. Research in ETW's laboratory is currently supported by the National Institutes of Health (1SC1GM081160-01), Professional Staff Congress–City University of New York, and New York State. Funding has also been obtained from the National Science Foundation, the U.S. Department of Defense, the U.S. Department of Agriculture, the Rockefeller Foundation International Program in Rice Biotechnology, the Rockefeller Foundation Biotechnology Career Fellowship Program, the American Cancer Society, and the McKnight Foundation Plant Biology Program.

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