

## Metabolic Engineering of Carotenoid Pathways in Crop Plants

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### Abstract

Carotenoids are plastid-synthesized and localized lipid-soluble C<sub>40</sub> tetraterpenoids universally distributed in the plant kingdom. These widespread pigments are integral and essential components of photosynthesis. Carotenoids are essential for photoprotection of plants which functions during photosynthesis and serving as precursors for the biosynthesis of the abscisic acid (ABA). Carotenoids are also very significant nutraceutical components of the animal diet and serving as precursor of vitamin A. Both mevalonate dependent and independent pathways for the formation of isopentenyl diphosphate are known. Genes encoding enzymes required for the biosynthesis of carotenoids have been identified. Even though most of the carotenoid biosynthetic genes have been cloned and identified, some aspects of carotenoid formation and manipulation in higher plants especially remain poorly understood. The topics of current interest are the progress and possibilities of metabolic engineering of plants to alter carotenoid content and composition in order to enhance the carotenoid content of plants to a level that will be required for the prevention of diseases which is a current need for research in both the basic and the applied aspects.

**Keywords:** Carotenoid biosynthesis; Isomerization; Lycopene;  $\beta$ -carotene; Metabolic engineering

### Introduction

Flower color is of paramount importance in plant biology. Three major groups of pigments—betalains, carotenoids and flavonoids—are responsible for the attractive display of flower colors. Carotenoids, the colored pigments ranging from yellow, orange to deep red, are biosynthesized by all photosynthetic bacteria, cyanobacteria, higher plants and also by some non-photosynthetic bacteria, fungi, and yeasts. More than 600 carotenoids are characterized structurally and the number is increasing continuously as newer compounds are being discovered. Commercially, carotenoids are used as colorants for human food and nutritional supplements, as feed additives to enhance the pigmentation of fish and eggs, as pharmaceutical products, and in the agriculture and cosmetic industry [1]. The major function of carotenoids in plants is in photosynthesis where they protect the photosynthetic apparatus from excess light. In recent years there has been considerable interest in the dietary carotenoids due to their provitamin A activity [2,3], high antioxidant potential [4] and their ability to prevent the onset of certain cancers [5,6], as well as age-related macular degeneration [7]. The beneficial role of carotenoids in maintaining human health, their important role in plant photo protection and their versatile usage as food and feed supplements make them potential candidates for enhancement and manipulation. Over the past three decades advances in molecular genetics and biotechnological approaches have led to the understanding of carotenoid biosynthesis and its manipulation in microorganisms and higher plants. The current knowledge of the molecular biology of carotenoids derives primarily from the study of the pathway in specific organisms, including the photosynthetic prokaryotes, Rhodospirillum rubrum and Synechococcus, bacteria of the genus Erwinia, the fungi Neurospora and the higher plants Zea mays (corn), Lycopersicon esculentum (tomato) and Capsicum annuum (pepper). Even though the structural genes of carotenoid biosynthesis have been identified and cloned, the regulation of carotenoid biosynthesis pathway is poorly understood. Therefore, the type and amount of carotenoids to be accumulated by transformation is still unpredictable which can be attempted by metabolic engineering in various crop plants with future research directions.

### Carotenoid Biosynthesis

The biosynthetic pathways involved in carotenoids formation were elucidated in the middle of the last century using various classical

biochemical and mutational studies [8]. Various modern molecular and biochemical techniques have facilitated functional complementation of genes leading to the creation of transgenic plants. These studies have enhanced the knowledge of carotenoid biosynthesis, its regulation, and the enzymes involved in the pathway [9].

### Formation of carotenoids

Originally it was believed that all isoprenoids were produced by using mevalonate (MVA) as a precursor of IPP, which is synthesized from acetyl-CoA via mevalonic acid [10] but later, the MVA-independent pathway for the formation of IPP was also discovered, which involves 1- deoxy-D-xylulose-5-phosphate (DXP) [11] (Figure 1). Eukaryotes, with the exception of the photosynthetic eukaryotes, use the MVA pathway for the isoprenoid synthesis [12-14].

### Metabolic engineering in crop plants

Even though conventional plant breeding approaches have increased the productivity successfully, the advantages of genetic engineering over this method include the ability to transfer genes in a faster and targeted manner. Carotenoid profiling of the target crop will help in selecting the gene(s) for metabolic engineering. Metabolite/precursor pool sizes, enzyme activities and location, gene expression profiles, carotenoid catabolism, interaction with other isoprenoid pathways, and regulatory mechanisms influence the choice and combination of genes and promoters necessary to manipulate the pathway. Currently amplification of the enzyme with the highest flux control coefficient or the “rate-limiting” enzyme is the principal target for carotenoid manipulation. Increasing flux through the pathway (quantitative engineering) seems to be promising for increasing the end product. Changing the composition of carotenoids or creating a new carotenoid in the tissue of interest (qualitative engineering) is the other objective of

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white varieties do not have pro-vitamin A content as they possess *Psy* allele [21]. Transgenic maize with enhanced pro-vitamin A in kernels were generated by overexpression of phytoene synthase (*Crt B*) and phytoene desaturase (*Crt I*) from *E. herbicola* under the control of  $\gamma$ -zein promoter. A 34-fold increase in total carotenoids was observed with preferential accumulation of  $\beta$ -carotene in endosperm [22].

**Wheat:** Cong et al., [23] carried out studies to increase the total carotenoid content in elite wheat (*Triticum aestivum* L.) EM12. EM denotes Embrapa, a Brazilian Agricultural Research Corporation which has developed hundred new cultivars for different regions of Brazil. Transgenic wheat was generated by expressing maize *y1* gene encoding phytoene synthase under endosperm specific *1Dx5* promoter along with phytoene desaturase (*Crt I*) gene from *E. uredoovora* under constitutive *CaMV 35S* promoter. The transgenic wheat showed light yellow colored endosperm and a 10-fold increase in the total carotenoid content was observed as compared to nontransgenic EM 12 variety.

Different software applications are now employed in the field of metabolic engineering. These applications support a wide range of experimental techniques. Computational tools are utilized throughout the metabolic engineering of particular pathway to interpret relevant information from large data sets, to present complex in a more manageable form, and to propose efficient network design strategies. There are large numbers of tools that can assist in modifying cell-based metabolic networks. Carotenoid pathway enzymes from different sources exhibit capacities in main carotenoid biosynthesis. A two-fold higher lycopene production is obtained in *E. coli* by the expression of carotenogenic enzymes from *P. agglomerans* (27 mg/L) than from *P. ananatis* (12 mg/L) [24]. Metabolic engineering allow the assembly of genes from different organisms for production of building new carotenoids [25].  $\beta$ -carotene production has been improved by hybrid expression of carotenogenic genes from *P. agglomerans* and *P. ananatis* in *E. coli* [26]. Mainly, a sufficient precursor supply is a prerequisite for high-yield production of carotenoids. Overexpression of the rate-limiting enzymes, 1-deoxy-D-xylulose-5-phosphate synthase and reductoisomerase led to a 3.6-fold increase in lycopene production in *E. coli* when compared with the native MEP pathway for IPP and DMAPP supply [27].

A great effort in metabolic engineering of the central carotenoid building block pathway is the introduction of a hybrid MVA pathway of *Streptococcus pneumonia* and *Enterococcus faecalis* into *E. coli*, which enables the recombinant host to produce 465 mg/L of  $\beta$ -carotene [28]. With more available genetic tools, microbial organisms such as *Pseudomonas putida* and *Bacillus subtilis* have also been developed as platform hosts for carotenoid production [29]. Carotenoids synthesis involves multiple enzymes [30]. A random approach is screening of the best orchestra from numerous combinatorial assemblies of required genes and control elements. BioBrick™ paradigm is capable of rapidly assembling a biosynthetic pathway in a variety of gene orders from different promoters in plasmids with different copy numbers [31]. It is possible to build a hybrid carotenoid pathway where each enzyme possesses a right turnover number, however, BioBrick™ assembly is still not in a high throughput to create vast combinatorial expression constructs for the best combination of carotenogenic genes. Recently, several advanced assembly methods using homologous recombination, such as sequence and ligation-independent cloning (SLIC), Gibson DNA assembly and reiterative recombination, have been applied to construct multigene circuits [32]. These advances promise to randomize all genetic components, including genes, promoters, ribosome binding sites, and other control modules to build a large number of individual genetic circuits for screening purposes. A so-called “randomized

BioBrick assembly” approach has been applied to the optimization of the lycopene synthesis pathway wherein the expression construct was designed to independently express each enzyme from its own promoter, which resulted in an increase by 30% in lycopene production [33]. A longer and more complicated pathway can be modularized into subsets, which contain pathway enzymes with similar turnover numbers. Modulating these subsets would be more convenient and efficient than regulating all components of the entire pathway for improved production [34]. The diffusion of pathway intermediates can decrease the effective concentrations of intermediates for following enzyme reactions and some intermediates may serve for competing pathways. By learning from Mother Nature, synthetic biologists spatially organize enzymes of the MVA pathway by protein scaffolds in *E. coli* to minimize diffusion limitation and achieve a 77-fold increase in mevalonic acid production [35]. Some intermediates of carotenoid synthesis such as isoprenyl diphosphates are toxic when they accumulate over the concentration threshold [36]. To avoid the accumulation of toxic intermediates, genetic sensors can potentially be coupled with gene expression cassettes to regulate the intermediate flux in a dynamic manner.

The rapid proliferation of genome-scale data for plants and other organisms makes it possible to systematically study diverse cellular processes. As heterogeneous high-throughput data sets have been acquired from different technologies in the “omics” fields, such as genomics, transcriptomics, proteomics, and metabolomics, it has become necessary to develop computational tools that can integrate and analyze them efficiently [37]. Microarrays and recently emerged RNA-Seq technology have proven to be crucial tools in producing transcriptional data sets by simultaneously detecting the expression of thousands of genes [38]. In recent years, new functional annotations of genes have been added to diverse biological networks, including regulatory networks, protein-protein interaction networks, and metabolic pathways. Despite these advances, dynamic behaviors of genes in specific pathways under specific conditions are still largely unexplored. Thus, in addition to the integration of heterogeneous data sources, analysis of them under the context of pathways is regarded as an essential step for functional studies of a complex biological system. In this type of analysis, transcriptomic data are normally mapped onto specific metabolic pathways to investigate the coordinated behavior of a set of genes. Developing efficient tools for this type of analysis is important in systematically characterizing and understanding the dynamics of biochemical pathways through utilization of multilevel information. As detailed information of biological pathways has been developed, both experimentally and computationally, more complete and precise pathways have been mapped. Currently, the representative biochemical pathway databases include MetaCyc and the Kyoto Encyclopedia of Genes and Genomes (KEGG); MetaCyc contains experimentally verified metabolic pathway and enzyme information curated from the scientific literature as well as computationally predicted metabolic networks for more than 1,600 different organisms [39]. KEGG is a knowledge base in terms of the network of genes and molecules resulting from their activities [40]; [41]. These databases are the primary resources that can be utilized to understand how genes and molecules are connected in biochemical pathways. Moreover, they can be combined with new resources or technologies for genomic and functional analysis, making it possible to expand previous databases and obtain increased depth and range of functions. For example, the database EGENES was developed to place genomic information, including ESTs of many plant species, into metabolic pathways and was integrated into the KEGG suite of databases [42]. Plants have evolved the ability to synthesize a large variety

of metabolites to protect themselves against various attacks and to attract flower pollinators. The regulation of metabolite biosynthesis is coordinated by specific transcription factors [43]. Bioinformatics analysis has indicated that genes within the same pathway, especially those clustered together in the pathway structure, are usually highly coexpressed [44]. This implies that those genes might be regulated by common transcription factors. Experimental evidence also supports that a subset of genes in the same pathway could be regulated by common transcription factors [45-47].

## Conclusion

The fundamental reaction sequences involved and the genes responsible for carotenoid biosynthesis have been isolated and characterized in several laboratories [48]. Successful enhancement in the carotenoid content has been achieved by various research groups by increasing plastid number/size [49], extraplastidial biosynthesis [50], modification of intracellular storage [51], modification of carotenoids synthesized like esterification [52], synthesis in unconventional plants/ plant parts [15], and the removal of feedback inhibition. The enhanced accumulation of provitamin A content may provide, at least partially, a solution to overcome vitamin A deficiency and related health problems like blindness, xerophthalmia, and growth retardation in developing countries, especially among children. The problem of delivering vitamin A orally in high risk countries where VAD (Vitamin A deficiency), leading cause of preventable blindness in children is prevalent may be resolved by supplementing their staple food such as rice with biofortified vegetables such as potato and tomato. Assuming daily requirement of vitamin A as 300 µg retinol equivalents/day for children and 800 µg for adults, and a conversion ratio of β-carotene into retinol of 6:1 used in human supplementation studies 100% of vitamin A requirement for children and 38% for adults can be met by approximately 5 g/day transgenic canola oil 60 g/day golden rice and 150 g/day golden potatoes [53].

In spite of the significant progress in elucidation of carotenoid biosynthesis in plants, its various aspects, especially metabolic fluxes leading to their accumulation, are yet to be understood to improve our capability to manipulate carotenoids in crop plants. The challenges faced in increasing the carotenoid concentration to higher levels is due to our limited knowledge regarding mechanisms and signals controlling plant carotenogenesis and cross-talk between metabolic pathways, which makes it difficult to predict the outcome of biotechnological approaches. Development of isoprenoid profiling methodologies and microarray approaches, and studies on mutants with respect to regulatory mechanisms that control carotenoid accumulation in chromoplasts of non-photosynthetic systems will provide additional insight into these mechanisms. Mapping analysis of quantitative trait loci (QTL) to identify genes responsible for carotenoid accumulation and transcript profiling of gene expression in carotenoid accumulating tissues and organs [54], might throw an additional light in identifying the potentiality of regulatory genes. All this knowledge will be helpful in designing and developing transgenic crop plants with carotenoid content tailored to the needs of a specific end group.

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