

Production of carotenoids by recombinant DNA technology

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Abstract Biotechnology can be used to effect the production of carotenoids by natural organisms. Algae and yeast strains have been developed by classical strain improvement methods to produce several different carotenoids. However, the carotenoid productivity of these organisms is low compared to carotenoid production by chemical synthesis. One way to increase the production of carotenoids in biological systems is to use recombinant DNA techniques. The carotenoid biosynthetic pathway is especially amenable to manipulation by recombinant DNA techniques since all carotenoids share a common precursor. Effecting these key steps with genetic engineering methods can increase carotenoid productivity. In addition natural systems have a very widespread biosynthetic capability, and these systems can be used to synthesize novel carotenoids for particular applications. Recently, many genes coding for enzymes catalyzing specific steps in the biosynthesis of carotenoids have been isolated and characterized from bacteria, cyanobacteria, and higher plants. This paper will focus on the application of carotenoid biosynthetic genes to affect carotenoid production in bacteria and yeasts.

INTRODUCTION

Carotenoids are produced by a diverse group of organisms, ranging from bacteria to higher plants. Over 600 different carotenoids have been identified from natural sources. This biological diversity and synthetic capability have not been exploited to develop systems for the production of carotenoids. Currently only β -carotene and astaxanthin are produced from biological sources and sold widely to the food and feed industries.

The key limitation to the use of biological systems to produce carotenoids is the low productivity of these systems compared to chemical syntheses. One way to increase the productivity of carotenoid synthesis is to use recombinant DNA technology. However, before recombinant DNA technology and genetic engineering can be used to increase the productivity, genes encoding the carotenoid biosynthetic enzymes need to be isolated and characterized.

Until very recently, genes for the carotenoid biosynthetic enzymes had not been isolated. This situation has changed markedly over the past three years. Currently a number of the genes coding for carotenoid biosynthetic enzymes have been isolated from bacteria, fungi, cyanobacteria, green algae, and higher plants (Table 1). With the large number of genes now available for further study, systems can be tested to increase the production of carotenoids from biological sources. Results from these studies will fuel the development of processes for the production of carotenoids from a number of different host organisms and the full range of carotenoid compounds from biological sources will be accessible.

The following will describe the characterization of carotenoid genes from *Erwinia herbicola* and the use of these genes to effect carotenoid synthesis in different host cells.

Table 1. Genes isolated from different organisms coding for carotenoid biosynthetic enzymes

| <u>Organism</u> | <u>GGDP synthase</u> | <u>Phytoene synthase</u> | <u>Phytoene desaturase</u> | <u>Zeta-carotene desaturase</u> |
|--------------------------|-------------------------|-------------------------------------|-------------------------------|---------------------------------|
| <i>Rhodobacter</i> | crtE (ref.1) | crtB (ref.1) | crtI (ref.1,2) | |
| <i>Erwinia herbicola</i> | crtE (ref. 3,4) | crtB (ref. 3,4) | crtI (ref. 3,4) | |
| <i>uredovora</i> | crtE (ref. 5) | crtB (ref. 5) | crtI (ref. 5) | |
| <i>Neurospora</i> | al-3 (ref. 6) | al-2 | al-1(ref. 7) | |
| <i>Synechocystis</i> | | | pds (ref. 8) | |
| <i>Synechococcus</i> | | pys (ref. 9) | pds (ref. 10) | |
| <i>Anabaena</i> | | | | zds (ref. 11) |
| <i>Dunaliella</i> | | | pds (ref. 10) | |
| Tomato | | pTOM5 (ref. 12,13) pys (ref. 14) | | |
| Soybean | | | pds (ref. 15) | |
| Pepper | GGDPS (ref.16) | pys | pds (ref. 17) | |
| Maize | | y1 (ref. 18) | | |
| <u>Organism</u> | <u>Lycopene cyclase</u> | <u>B-Carotene hydroxylase</u> | <u>Zeaxanthin glycosylase</u> | |
| <i>Erwinia herbicola</i> | crtY (ref. 4) | crtZ (ref. 4) | crtX (ref. 4) | |
| <i>uredovora</i> | crtY (ref. 5) | crtZ (ref. 5) | crtX (ref. 5) | |

THE GENES FROM *ERWINIA*

Six genes coding for six carotenoid biosynthetic enzymes from the bacterium *E. herbicola* (ATCC 39368) were characterized. The carotenoid pathway is shown in Figure 1.

All these genes have been sequenced, and the sizes of the corresponding proteins have been determined. Table 2 gives a summary of the size of the genes as well as the size of the proteins. A number of manipulations have been performed with these genes to understand better the requirements for maximal activity of the gene.

Table 2. The size of the carotenoid biosynthesis genes and the size of the corresponding enzymes from *Erwinia herbicola*.

| <u>Carotenoid Gene</u> | <u>Gene Size (base pairs)</u> | <u>Protein Size (kilodaltons)</u> |
|-------------------------------|-------------------------------|-----------------------------------|
| GGDP synthase | 921 | 36 |
| phytoene synthase | 927 | 38 |
| phytoene desaturase | 1470 | 52 |
| lycopene cyclase | 1125 | 39 |
| β -carotene hydroxylase | 531 | 19 |
| zeaxanthin glycosylase | 1200 | 44 |

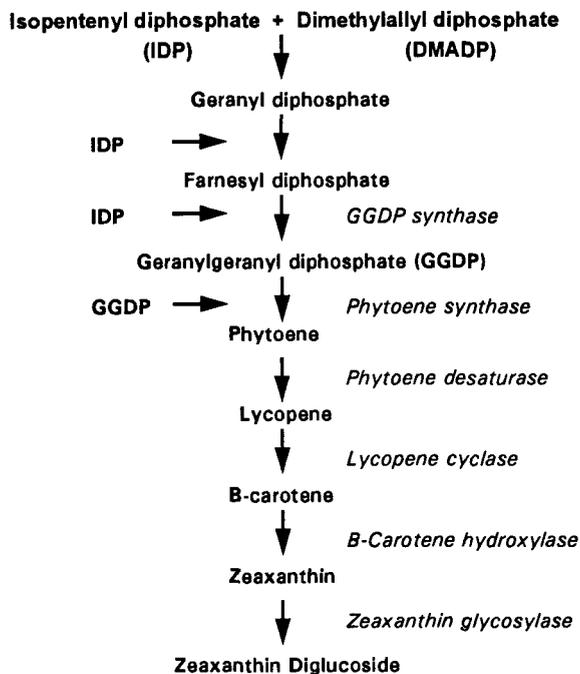


Figure 1. The biosynthetic pathway for carotenoid synthesis in *Erwinia herbicola*. Farnesyl diphosphate is converted to geranylgeranyl diphosphate by the enzyme GGDP synthase. A single enzyme, phytoene synthase, combines two molecules of geranylgeranyl diphosphate to form the first C₄₀ carotenoid, phytoene. Phytoene is converted to lycopene by a single enzyme, phytoene desaturase. β -Carotene is formed from lycopene in a reaction catalyzed by the enzyme lycopene cyclase. Two hydroxyl groups are added at the 3 and 3' positions of β -carotene to form zeaxanthin in a reaction catalyzed by β -carotene hydroxylase. Finally, two molecules of glucose are added to zeaxanthin to form zeaxanthin diglucoside in a reaction catalyzed by zeaxanthin glycosylase.

The gene for GGDP synthase was analyzed in detail. The N-terminus of GGDP synthase is shown in Fig. 2a. The gene for GGDP synthase was engineered for expression in an *E. coli* expression system and, following enzymatic analysis, the gene was found to encode a protein with a specific enzyme activity of 6.35 nmol/min/mg-protein. To understand better the role of the N-terminus region of the enzyme for activity, the first 17 amino acids of the protein were deleted from the gene. This version was then introduced into the *E. coli* expression system, but was found to be completely inactive. To determine the function of the first 17 amino acids in the activity of the protein a number of different manipulations were performed in this region. Surprisingly, it was found that the deletion of the first 13 amino acids of the protein and replacement with four novel amino acids caused an increased specific activity of the protein to 12.15 nmol/min/mg-protein when this version was placed in the *E. coli* expression system (Fig. 2b).

a)
 1 5 10 15
Met Val Ser Gly Ser Lys Ala Gly Val Ser Pro His Arg Glu Ile Glu Val ...

b)

Met Ala Glu Phe Glu Ile Glu Val ...

Figure 2. N-terminal sequence of the gene for GGDP synthase from *E. herbicola* and the N-terminal sequence of a more active form of the gene.

The C-terminus of the protein for GGDP synthase was also examined and the deletion of the last 14 amino acids of the protein caused an increase in specific activity to 23.28 nmol/min/mg-protein. In summary the manipulation of both the 5' and the 3' ends of the gene led to a version of GGDP synthase that had about a four-fold increase in specific activity of the protein. The most active version of the gene was used in all subsequent manipulations to affect carotenoid synthesis in new host cells.

The gene for lycopene cyclase was also examined in detail. In Fig. 3 is shown the 5' end of the gene and the corresponding amino acid sequence. The initiation codon of the gene is GTG, instead of the much more common ATG. Nevertheless many bacteria can recognize GTG as an initiation codon, and the lycopene cyclase gene was efficiently transcribed and translated, and the protein functioned properly in *E. herbicola*, *E. coli*, and *Agrobacterium tumefaciens*.

| | | | | | | | | | | | |
|------------|------------|------------|------------|------------|------------|----------------|--|--|--|--|--|
| 1 | | 5 | | | | | | | | | <u>Activity</u> |
| GTG | AGG | GAT | CTG | ATT | TTA | GTC... | | | | | <i>E. coli</i>, <i>E. herbicola</i> |
| Met | Arg | Asp | Leu | Ile | Leu | Val ... | | | | | <i>A. tumefaciens</i> |

Figure 3. N-terminal nucleotide sequence and amino acid sequence of lycopene cyclase from *E. herbicola*. The native version of the gene with the GTG initiation codon is recognized by a number of different bacteria.

Since there was a desire to introduce the gene for lycopene cyclase into eucaryotic organisms, which do not recognize GTG as an initiation codon, the beginning of the gene was changed to introduce an Nco I restriction site into the gene. This manipulation changed the GTG initiation codon to ATG, but it also changed the second amino acid from an Arg to a Gly residue. This version of the gene and corresponding protein functioned properly in *E. coli*, but the enzyme was completely inactive when it was transferred to *Saccharomyces cerevisiae*. A second manipulation was performed to change the initiation codon to ATG but at the same time retain the second amino acid as an Arg residue. An Sph I restriction site was introduced at the beginning of the lycopene cyclase gene. This converted the GTG initiation codon into an ATG initiation codon and also retained the Arg residue at the second position. This version of the gene coded for an active enzyme in *E. coli* as well as in *S. cerevisiae*.

To determine if these genes could be used to affect carotenoid synthesis in new host cells, they were introduced into two different organisms. The first is *Rhodobacter sphaeroides*, a phototrophic bacterium that produces carotenoids. In this organism phytoene is converted to neurosporene which is then further converted to spheroidene and spheroidenone. To determine if carotenoid synthesis can be affected by a foreign gene, the *E. herbicola* gene for phytoene desaturase was introduced into a mutant of *R. sphaeroides* that was blocked in the enzyme that converts phytoene to neurosporene.

The *E. herbicola* phytoene desaturase forms lycopene from phytoene, and small amounts of lycopene were found in the transformed *R. sphaeroides* cells. However, two new compounds were also produced that were identified as spirilloxanthin and 2-ketospirilloxanthin (Figure 4). Lycopene that was produced from the action of the *E. herbicola* phytoene desaturase apparently was further converted by the endogenous carotenoid enzymes that normally convert neurosporene to spheroidene and spheroidenone. These experiments indicate that the use of recombinant DNA technology can alter the production of carotenoids in organisms that normally produce carotenoids. These experiments also indicate that novel carotenoids can be synthesized through the use of recombinant DNA technology.

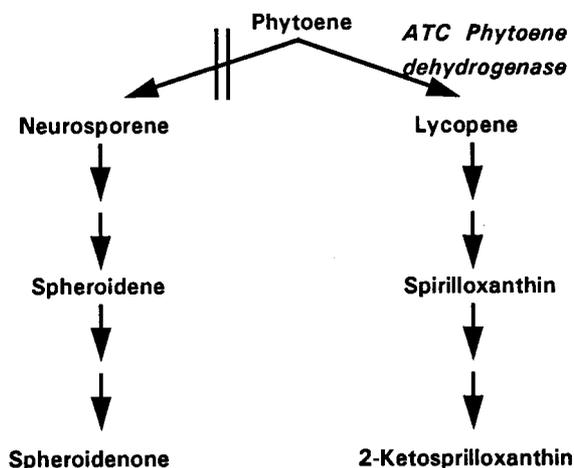


Figure 4. Effect of the introduction of the *E. herbicola* gene for phytoene desaturase into a strain of *Rhodobacter sphaeroides* that accumulated phytoene due to a mutation in the phytoene desaturase gene for the conversion of phytoene to neurosporene.

To determine if carotenoids could be produced in an organism that does not normally produce carotenoids, the genes for carotenoid synthesis were introduced into *S. cerevisiae*. *S. cerevisiae* contains an active sterol biosynthetic pathway and the key intermediate in this pathway is farnesyl diphosphate. The genes for GGDP synthase and phytoene synthase were added to *S. cerevisiae* cells, and the production of phytoene was confirmed. Then the genes for phytoene desaturase, lycopene cyclase, and β -carotene hydroxylase were individually added to the cells. The result is that all these enzymes function properly in *S. cerevisiae*, and strains were developed that produced phytoene, lycopene, β -carotene, and zeaxanthin. These experiments demonstrate that carotenoids can be produced in organisms that do not normally produce carotenoids. In addition these experiments demonstrate that entire biosynthetic pathways can be introduced into new host cells through recombinant DNA technology.

Biological systems offer a new production vehicle for the synthesis of carotenoids. As shown above the use of recombinant DNA technology can be used to effect and increase the production of carotenoids in a variety of organisms. The further application of recombinant DNA technology to carotenoid synthesis will broaden the range of possible compounds produced by biological sources.

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