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Carotenoid Biosynthesis and Its Regulation in Filamentous Fungi

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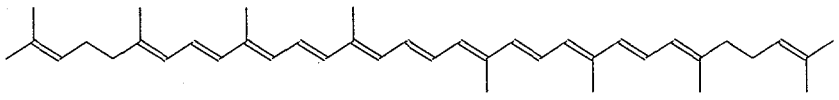
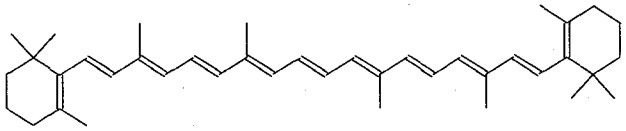
Structure of carotenoids

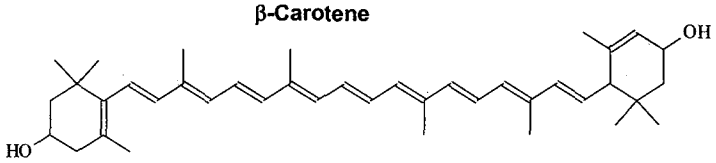
Carotenoids are isoprenoids that are common to all photosynthetic tissues and many microorganisms including filamentous fungi. They are divided into the hydrocarbon carotenes, such as lycopene and β -carotene or xanthophylls, typified by lutein (Fig. 1). They generally consist of eight isoprene units joined together so that the linking of units is reversed at the centre of the molecule. The most obvious feature of the carotenoid molecule is the polyene chain, which may extend from 3 to 15 conjugated double bonds, thereby producing colours from yellow to red. The length of the chromophore determines the absorption spectrum of the molecule, its colour to the eye and allows structural identification by spectroscopy (Fraser et al., 2000).



Lycopene



β-Carotene



Lutein

Fig. 1 Structures of typical carotenoids

Biosynthesis of carotenoids

A schematic overview of the isoprenoid biosynthetic pathway (Fig. 2) reveals the large number of intermediates, the varied nature of the end products and also the highly branched nature of the pathway

itself. In addition, the branches of the pathway are located in different subcellular compartments. For example, membrane-bound enzymes catalyse the steps beyond phytoene formation. Taken together, the branched nature of the pathway and multiple subcellular locations suggest a sophisticated series of regulatory interactions, with the coordinated flux of isoprenoid units into each branch of the pathway. Thus, the likelihood of metabolic cross talk is high, and perturbations of the flux by genetic engineering may result in pleiotropic effects on metabolite levels. Further details of the biosynthetic pathways can be found in Fraser and Bramley (2004).

Cytosolic enzymes, apart from HMG CoA reductase that is located on the endoplasmic reticulum, catalyse the early steps, leading to phytoene formation. The desaturation, cyclisation and oxygenation reactions leading to end products are all catalysed by membrane bound enzymes. Most of these have not been purified from fungi; although phytoene dehydrogenase from *Phycomyces blakesleeanus* was purified some years ago (Fraser and Bramley, 1994) and is a multimeric protein (Sanz et al., 2002). Several species of fungi have been used *in vitro* to study the biochemistry of the reactions (e.g. El-Jack et al., 1987; Fraser et al., 1991). Similarly, not all of the genes have been cloned, although those that have are shown in Figure 2. One feature in several species is a single gene for phytoene synthase and lycopene cyclase (Velayos et al., 2000; Arrach et al., 2001, Rodriguez-Saiz et al., 2004). Unfortunately, there is no uniform nomenclature for carotenoid genes, as shown in this figure.

The cyclisation of lycopene creates a series of carotenes that have one or two rings of either the β- or ε-type. Lycopene β-cyclase (LCY-B/CRTL-E) catalyses a two-step reaction that leads to β-carotene (two β-rings), whereas lycopene ε-cyclase (LCY-E/CRTL-E) creates one ε-ring to produce δ-carotene. It is assumed that α-carotene is formed by the action of both enzymes. The cyclisation can also proceed via β-zeacarotene (Bramley et al., 1977). Xanthophylls are formed by the oxygenation of carotenes, typically by the addition of hydroxyl, epoxy or keto groups. Hydroxylation at 3C and 3C' positions is carried out by two types of enzymes; one specific for β-rings and one for ε-rings. Astaxanthin formation also requires the introduction of two carbonyl groups.

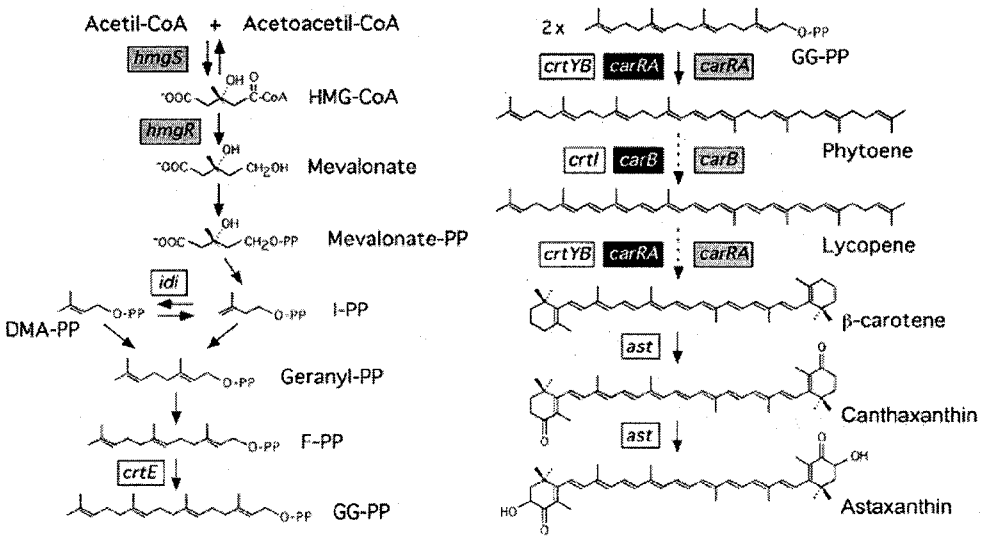


Fig. 2 The carotenoid biosynthetic pathway (Avalos et al., 2005)

Left: early steps of the terpenoid pathway leading to GGPP. Right: biosynthesis of carotenoids from GGPP. The dotted arrows represent more than one reaction. The genes available in *Xanthophyllomyces* (white boxes), *Blakeslea* (grey boxes) and *Phycomyces* (black boxes) are indicated close to the reactions carried out by the corresponding encoded enzymes. The synthesis of astaxanthin from β-carotene is only present in *Xanthophyllomyces*.

Biological and nutritional importance of carotenoids

Coloured carotenoids are found in fruits, flowers and roots, where they probably act as attractants to pollinators and for seed dispersal. In the chloroplast they participate in light harvesting in photosynthetic membranes and also protect the photosynthetic apparatus from excessive light energy by quenching triplet chlorophylls, superoxide anion radicals and singlet oxygen. The role of carotenoids in fungi and other non-photosynthetic organisms is less well established. They may have a role in photoprotection and as singlet oxygen quenchers (Gessler et al., 2002).

Animals are unable to synthesise carotenoids *de novo*, and so rely upon the diet as the source of these compounds. Dietary carotenoids fulfil essential requirements for human and animal nutrition. β-Carotene is the most potent dietary precursor of vitamin A, the deficiency of which leads to xerophthalmia, blindness and premature death (Mayne, 1996).

Commercial value of fungal carotenoids

The interest in the biological production of carotenoids is stimulated by the current trend against chemical food additives, the scarcity of β-carotene in industrial foods and the increasing demand for carotenoids in animal feeds. Fungi are a traditional source of the fermentation industry for the production of metabolites of applied interest. Rapid growth, ease genetic manipulation and a rich secondary metabolism are some of their notable advantages.

The fungi used by industry for the production of β-carotene and astaxanthin, *Blakeslea* and *Xanthophyllomyces*, have been the subject of considerable research and development by fermentation technologists (Ciegler 1965; Ninet and Renaut 1979; Fang and Cheng 1993; An et al. 1996; Yamane et al. 1997). *Blakeslea* belongs to a broad taxonomic group, the Mucorales, in which β-carotene biosynthesis is a common trait. Other Mucorales, such as *Phycomyces blakesleeanus* (reviewed by Cerdá-Olmedo, 1987, 1989) and *Mucor circinelloides* (Navarro et al. 1995; Fraser et al. 1996; Ruiz-Hidalgo et al. 1997; Velayos et al. 1997), have not been used for industrial production but are more amenable to genetic and molecular analysis and have been a major source of information on the genetics and biochemistry of the biosynthetic pathway. Genetic research in *Blakeslea* has been limited to the description of mutants for sexual activity (Khrabrova and Zhdanov 1979) and carotenoid production (Mehta and Cerdá-Olmedo 1995). Strains with increased carotenoids have been reported (Mehta and Cerdá-Olmedo 1995; An et al., 1989, 1991; Fang and Cheng 1992, 1993) suggesting that the development of carotenoidhyperproducing strains is feasible.

Canthaxanthin and astaxanthin, extensively used by poultry and fishery industries. The main source for astaxanthin is the yeast *Xanthophyllomyces dendrorhous,* formerly *Phaffia rhodozyma* (Johnson 2003). Other fungi produce other carotenoids that, to date, have not found a commercial application.

Regulation of carotenoid formation in fungi

A variety of factors have been shown to alter the formation and accumulation of carotenoids in filamentous fungi. These include:

·Light

·Bioinducers such as trisporic acid

·Miscellaneous synthetic chemicals

The factors that affect carotenogenesis in fungi have been known for many years (reviewed by Bramley and Mackenzie, 1988), but it is only in more recent years that the underlying molecular mechanisms have started to be elucidated.

1. Light

Photoregulation of carotenoid biosynthesis has been reported in at least 15 species of fungi. In some cases, light enhances carotenogenesis, e.g., *Phycomyces* (Cerdá-Olmedo, 2001), whilst in other such as *Aspergillus giganteus* (El-Jack et al., 1987) there is strict photoregulation of carotenogenesis. In *Blakeslea* there are reports of both inhibition by light (Sutter, 1970) and stimulation (Quiles-Rosillo et al., 2005). Blue light signalling chains have been found in *Phycomyces* that include protein phosphorylation (Tsolakis et al., *1999).* There have been many studies on mutants of *Phycomyces* in which induction of carotene biosynthesises by light is defective (Cerdá-Olmedo, 2001). It is likely that the mechanisms for light regulation are conserved between closely related species such as *Blakeslea, Phycomyces* and *Mucor.*

2. Bioinducers

Mated cultures and intersexual heterokaryons of the Mucorales (+ and - strains grown together, or containing both sexual determinants in different nuclei of the same mycelium) contain increased amounts of carotenoids), e.g. *Blakeslea* and *Phycomyces* (Mehta and Cerdá-Olmedo, 2003; Murillo and Cerdá-Olmedo, 1976). It is believed that the increase is due to induction of carotenogenesis by trisporic acids, especially trisporic acid C. These are formed from β-carotene by oxidative cleavage, but the detailed pathway remains to be elucidated (Gessler et al., 2002).

3. Miscellaneous chemicals

A large number of chemicals are known to either inhibit or stimulate carotenoid formation in fungi. Chemical activators in *Blakeslea* and *Phycomyces* include α- and β- ionone and retinol (Cerdá-Olmedo, 1989)

Mechanisms of regulation

1. Transcriptional control

Several reports have shown that up or down-regulation of gene expression occurs in response to light, chemicals and developmental stage of the fungus. For example, the phytoene dehydrogenase gene of *Phycomyces* is up-regulated by blue light and vitamin A had a similar effect (Ruiz-Hidalgo et al., 1997). Similarly, GGPP synthase gene (*carG*) of *Mucor circinelloides* is also up-regulated by blue light. This gene has several APE-like sequences in the promoter region, which participate in the blue light regulation of the expression of different fugal genes (Velayos et al., 2003). Carotene over-producing mutants contain more HMG CoA reductase mRNA than the wild type (Ruiz-Albert et al., 2002). The *crgA* gene from *Mucor circinelloides* has a dominant-positive effect on light regulation of carotenogenesis (Navarro et al., 2000). Many mutants of *Phycomyces* have been isolated with significant changes in carotenoid content, as a result of mutations in regulatory genes (Cerdá-Olmedo, 2001).

2. Feedback regulation and sequestration

Feedback regulation by β-carotene or one of its metabolites has been demonstrated in *Phycomyces blakesleeanus* (Murillo and Cerdá-Olmedo, 1976), but does not occur in *Blakeslea trispora* enough to explain the increase in carotene content. Instead, carotenoid overproduction involves the accumulation of carotene as crystal deposits, making unnecessary globule over accumulation. Thus, although lipid globules play an important role as storage mechanism, their amount is not critical. Lipid globules lack the ability to synthesise carotenoids from mevalonic acid, indicating a storage function for the globules independent of biosynthetic activity. Taken together, the results obtained indicate that under the industrial production conditions (submerged culture), lipid biosynthesis is not a bottleneck limiting the ability of *Blakeslea* to accumulate carotenoids. (Bramley, Kiano, Fraser, Avalos et al., unpublished).

3. Relationship between carotenoids and levels of carotenogenic enzymes in *Blakeslea*

Antisera to HMG-CoA reductase and separate domains of phytoene synthase and lycopene cyclase from *Phycomyces* and IPP isomerase from *Xanthophyllomyces* have been prepared. Western blots of carotenogenic enzymes in single and mated *Blakeslea* cultures of either wild type strains (F921 and F986) or mutants with elevated carotenoid content (SB32 and SB38) have been carried out. No phytoene synthase in 2-day old *Blakeslea* mycelia was found either with the wild type or with mutant strains. A weak signal corresponding to a protein of about 40 kDa was detected in wild type mated cultures. The signal was stronger with extracts from a mated SB32 / F986 culture and even stronger with extracts from a mated SB32 / SB38 culture, suggesting that the sexual stimulation results in enhanced enzyme levels. Experiments with older SB32 / F986 cultures showed an increase in the enzyme levels in the first days and a subsequent decrease between days 4 and 8. In vivo, β-carotene accumulated over the first 4 days and stayed constant afterwards, suggesting a correlation with phytoene synthase levels (Table 1).

Experiments with antisera against IPP-isomerase detected the enzyme, and showed an induction of the

Table I. Amount and activity of phytoene synthase in *Blakeslea* cultures in relation to their carotene content (Fraser, Kiano, Bramley, Breitenbach and Sandmann, unpublished).

	Phytoene synthase		
	Amount(μg/g protein)	Activity(dpm/mg protein h)	Carotene(mg/g dry wt)
Wild types			
F921 (-)	<20	nd	0.60
F986 (+)	106	<50	0.71
Mutants			
SB32 (-)	569	<50	1.66
SB38 (+)	708	nd	2.25
Mated cultures			
F921 + F986	248	1700	1.38
SB32 + SB38	2496	650	3.13
SB32 + F986	2111	950	9.20

protein level in the mated cultures of the wild type or the mutants compared with the corresponding single cultures. In contrast, no signals could be detected with HMG-CoA reductase antisera. As an alternative approach, the enzymatic activities were also investigated in vitro. ^{14}C-IPP, ^{14}C-MVA and ^{3}H-GGPP were used as radiolabelled precursors to study the incorporation into phytoene and β-carotene in cell extracts from mycelia grown in liquid or surface conditions. The major differences were found between single and mated cultures, the latter exhibiting a 5-10-fold increase in their biosynthetic phytoene and βcarotene activities. This result is in agreement with the ones obtained in western blot analyses.

Acknowledgments

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