# Photoregulation of Carotenoid Biosynthesis in Mutants of *Neurospora crassa*: Activities of Enzymes Involved in the Synthesis and Conversion of Phytoene

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Synthesis of carotenoids is photoregulated in many fungi including *Neurospora crassa*. In order to investigate the regulatory mechanism at the enzyme level, several carotenoid mutants of *Neurospora* were used to determine the activities of enzymes involved in the carotenoid biosynthetic pathway after growth under illumination or in darkness. Light stimulation of carotenoid formation was due to enhanced activities of three subsequent enzymes, geranylgeranyl pyrophosphate synthase, phytoene synthase, and phytoene desaturase indicating a coordinated regulation at the enzyme level. Farnesyl pyrophosphate synthase and lycopene cyclase were not involved in light regulation. Immunological studies showed that in the case of phytoene desaturase higher activity in the light originated from an increased amount of this enzyme in light-grown cultures.

## Introduction

Carotenoids are synthesized by photoautotrophic as well as by heterotrophic organisms. In fungi, photoregulation of this biosynthetic pathway is widespread (see ref. [1] and [2] for review). Many species form only low levels of carotenoids in dark-grown cultures. After illumination, a rapid increase of carotenoid production is observed. One of these species is *Neurospora crassa*. Detailed studies have been carried out on photoinduction of carotenogenesis [3], action spectra [4] and fluence response [5]. Some early cell-free studies indicated that various stages of the pathway leading to phytoene are photoregulated [6–8]. However, more detailed investigations at the level of single enzymes of the pathway leading to cyclic carotenes are still missing to answer the question of which of the enzymes are photoinducible and to what extent. They are now most actual as recent works on gene activation by light in *N. crassa* [9, 10] suggested a coordinated photoinduced synthesis of more than one enzyme of the carotenogenic pathway.

In the present publication, enzyme activities of single enzymes covering the whole carotenogenic pathway from dimethylallyl pyrophosphate (DMAPP) to phytoene and further on to desaturated colored carotenes were measured. As several carotenogenic mutants are available which target different steps in the pathway [11], they were employed in this study to facilitate determination of single enzymes. The results obtained allow the identification of several reactions which are all responsible for the stimulation of carotenogenesis by light.

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## Materials and Methods

The *Neurospora crassa* mutant strains al-2 (FGSC 313), al-3 (FGSC 2082) obtained from the Fungal Genetic Stock Center, University of Kansas, Kansas City, U.S.A. and YLO [12] as well as the *Fusarium* mutant SG4 [13] and the *Phycomyces* mutant C5 were grown under continuous light in a medium containing 2.4% potato dextrose broth. The mycelia were harvested after four days by filtration. They were used either directly to assay their carotenogenic activity or lyophilized for two days to be used for synthesis of $^{14}$C-labeled precursors and HPLC analysis.

Extracts were prepared for *in vitro* carotene biosynthesis from 1 g of freshly harvested *Neurospora* mycelium squeezed to dryness by adding 0.5 ml of 1 M Tris-HCl buffer, pH 8.0 containing 5 mM DTT and grinding with sand. The homogenate was centrifuged (10,000 × *g*) for 1 min and the supernatant collected.

Other fungal extracts were obtained from *Phycomyces* C5, *Fusarium* SG4 and *Neurospora* al-3

by rubbing the freeze-dried mycelia through a sieve (mesh size 0.4 mm) and mixing 0.2 g of the fine powder with 1.5 ml of 0.4 M Tris-HCl buffer, pH 8.0 containing 5 mM DTT. The supernatant after centrifugation (10,000 × *g*) for 10 min was further used.

The assay medium for determination of carotenogenic activity contained in 0.5 ml: 5 µmol ATP, 3 µmol $MnCl_2$, 2 µmol $MgCl_2$, 2 µmol FAD, and 200 µl of *Neurospora* enzyme extract. Radioactive substrates were as specified in Tables II and III either IPP (specific radioactivity 1.95 GBq/mmol) or farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP) or phytoene generated *in situ* with extracts from *Neurospora* al-3, *Fusarium* SG4 and *Phycomyces* C5, respectively, from [$^{14}$C]mevalonic acid (specific radioactivity 1.98 GBq/mmol). Incubation proceeded for 2 h at 30 °C. In case of FPP and GGPP synthase assays HCl was then added to a final concentration of 2 M to hydrolyze the prenyl pyrophosphates into their alcohols and the samples kept for 30 min at room temperature. When formation of radioactively labeled carotenes was measured, 2 ml of 60% KOH in methanol were added and heated for 15 min at 60 °C. The prenyl alcohols as well as the carotenes were partitioned into 10% diethylether in hexane. Finally, all the radioactively labeled compounds formed during the enzymatic reaction were separated by HPLC as their prenyl pyrophosphate alcohols on a 25 cm Spherisorb ODS-2, 5 µm column with acetonitrile/methanol/2-propanol (85:10:5, v/v/v) as eluent. Radioactivity was recorded by an on-line radioactivity monitor Ramona LS. Flow was 1 ml/min.

Freeze-dried *Neurospora* mycelium was extracted for carotenoid analysis by suspending 30 mg in 20 ml of methanol containing 6% KOH and heating for 20 min at 60 °C. After partitioning into diethylether, the organic phase was evaporated to dryness and resuspended in acetone. The carotenoids were separated and quantitated by HPLC using the same HPLC system as described above. Spectra were recorded from the elution peaks with a Waters 994 Programmable Photodiode Array Detector.

Western blots were carried out after SDS polyacrylamide gel electrophoresis. Homogenates of 5 mg of pulverized freeze-dried YLO mycelium from an illuminated or dark-grown culture in 0.5 ml 0.1 M Tris-HCl buffer, pH 8.0 containing 5 mM DTT, were boiled for 5 min with sample buffer and 10 µl were applied on 10% gels. The antiserum was raised against the phytoene desaturase from *Rhodobacter capsulatus*. For this purpose, parts of the crtI reading frame was overexpressed corresponding to a C-terminal protein fragment of 43 kDa which was purified by gel electrophoresis. The technical details were as previously described [14].

## Results

Before carrying out *in vitro* carotenogenic reactions, *N. crassa* mutant YLO was analyzed for its ability to synthesize various carotenoids in light- and dark-grown cultures. Fig. 1 shows a HPLC trace of an extract obtained from an illuminated YLO culture. Nine different carotenoids could be detected together with various *cis* isomers (numbers with prime) and their spectra recorded from

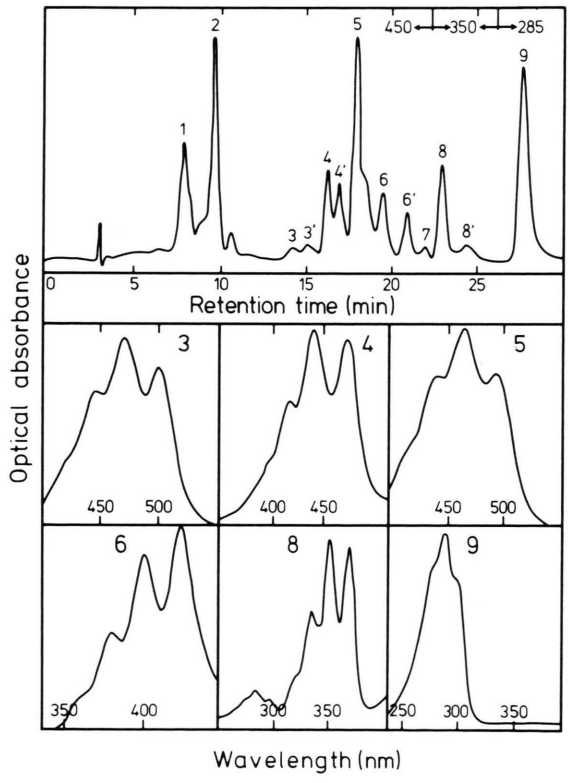


Fig. 1. HPLC separation of carotenoids accumulated by *Neurospora crassa* mutant YLO and selected absorbance spectra of *cis* isomers.

the elution peaks. Compounds **3** and **3'** were lycopene (all-*trans* and a *cis* isomer, respectively) as identified by reference compounds and by spectra. Pigment No. **1** exhibited the same spectrum and it can be regarded as a hydroxy derivative of lycopene due to its high polarity (= low retention time). Also for γ-carotene (No. **5**) a polar derivative with a similar spectrum (No. **2**) could be found. Pigments No. **4, 6** and **7** exhibited the typical spectra of neurosporene, ζ-carotene and β-carotene, respectively. Pigments No. **8** and **9** were identified as colorless phytofluene and phytoene. All these carotenoids are quantitated in Table I. Carotenoid production was 5 times higher in illuminated YLO cultures than those grown in darkness. In addition, more carotenes with a higher degree of desaturation were found in the illuminated culture (52%) as compared to the dark culture (29% of total carotenoids).

Table I. Carotenoid formation (μg/g d.w.) of *N. crassa* mutant YLO.

| Carotenoids | Light | Dark | L/D ratio |
|---|---|---|---|
| Phytoene | 118.7 | 34.1 | 3.5 |
| Phytofluene | 18.0 | 3.1 | 5.8 |
| ζ-Carotene | 39.1 | 3.1 | 12.6 |
| Neurosporene | 12.5 | 1.1 | 11.4 |
| β-Zeacarotene | 1.4 | <0.1 | 1.4 |
| Lycopene* | 12.2 | 2.4 | 5.1 |
| γ-Carotene* | 42.2 | 3.5 | 12.1 |
| β-Carotene | 4.2 | 1.0 | 4.2 |
| Sum | 248.3 | 47.8 | 5.2 |

* Including polar lycopene and γ-carotene derivatives, respectively.

Overall carotenogenic activity of the whole pathway was detected *in vitro* by using [$^{14}$C]IPP or [$^{14}$C]phytoene as substrates (Table II). With IPP, radioactivity was incorporated into all carotenes from phytoene to γ-carotene. Enzymatic formation of carotenes was about 4.6 times higher with material from the illuminated compared to dark-grown cultures. Conversion of phytoene into subsequent colored carotenes was very efficient in our preparations. Also [$^{14}$C]phytoene was converted to more desaturated carotenes by the membrane preparations with an almost 3-fold higher activity when an enzyme preparation from a light-grown culture was used.

Table II. *In vitro* biosynthesis of carotenes by light- and dark-grown mutant YLO from [$^{14}$C]IPP and [$^{14}$C]phytoene. Reaction was carried out for 2 h with YLO extracts equivalent to 3 mg of protein and $10^6$ dpm of [$^{14}$C]IPP (52.7 mCi/mmol) or $2 \times 10^4$ dpm of [$^{14}$C]phytoene.

| Carotenes | Radioactivity [dpm] incorporated from IPP | | from phytoene | |
|---|---|---|---|---|
| | Light | Dark | Light | Dark |
| Phytoene | 820 | 415 | 6759 | 13122 |
| Phytofluene | 244 | 105 | 1193 | 212 |
| ζ-Carotene | 1734 | 202 | 1591 | 610 |
| Neurosporene | 4612 | 582 | 3940 | 1296 |
| Lycopene | 948 | 348 | 1147 | 533 |
| γ-Carotene | 4715 | 1232 | 4774 | 1372 |

As desaturation of phytoene to lycopene is catalyzed by the product of one gene [15], a relative activity value which considers the number of introduced double bonds was estimated as an indicator of total desaturation activity in light- and dark-grown *Neurospora*. It was calculated from the radioactivities accumulated in the individual carotenes by (2 × ζ-carotene + 3 × neurosporene + 4 × lycopene + 4 × γ-carotene): 4 × (phytoene + ζ-carotene + neurosporene + lycopene + γ-carotene). Judging from the values of 0.53 and 0.18 an almost 3-fold higher desaturase activity can be estimated in the light-grown mycelia. Calculation of the rate of conversion of lycopene to γ-carotene (radioactivity in γ-carotene divided by the sum of radioactivity in γ-carotene and lycopene) gave values of about 0.8 for incubation with mycelia of either light- and dark-grown YLO. This indicates that lycopene cyclization in *Neurospora* is independent of illumination.

The activities of the three enzymes directly involved in the pathway leading to synthesis of phytoene was assayed in appropriate mutants grown in light or darkness (Table III). Mutant al-3 in which the conversion of FPP is blocked [8] was used to assay FPP synthase. In mutant al-2 GGPP conversion is defective. Therefore, it was used to determine GGPP synthase activity. Mutant al-1 has been characterized lacking phytoene desaturase activity, which makes it a very suitable mutant to assay phytoene synthase. The activities of both prenyl transferases, FPP synthase and GGPP synthase, were very high in *Neurospora* whereas enzymatic activity of phytoene synthase was lower. Comparing the rates for light- and

Table III. *In vitro* assay of farnesyl pyrophosphate (FPPS), geranylgeranyl pyrophosphate (GGPPS) and phytoene synthase (PS) using different albino (al) mutants grown in light or darkness. Substrates for the reactions were $5 \times 10^5$ dpm IPP (line 1), $2 \times 10^4$ dpm FPP (line 2) and $5 \times 10^4$ dpm GGPP (line 3). For the calculation of the conversion rate in line 1, radioactivity of the endogenously accumulated DMAPP was used.

| Mutant | Reaction | Activity as conversion rate Light | Dark | Ratio Light/Dark |
|---|---|---|---|---|
| 1. al-3 | DMAPP → FPP (FPP-S) | 86.4 | 82.0 | 1.1 |
| 2. al-2 | FPP → GGPP (GGPP-S) | 40.3 | 28.9 | 1.4 |
| 3. al-1 | GGPP → phytoene (P-S) | 14.9 | 6.6 | 2.3 |

dark-grown cultures, FPP synthase activity was more or less identical. The other two enzymes showed higher activities in illuminated cultures than in those grown in darkness. For GGPP synthase this increase was about 40%, and for phytoene synthase a 2.3-fold higher activity was determined.

Western blot experiments were carried out for phytoene desaturase to determine whether higher activities in the light correlate to a more abundant protein or to its activation. Several antisera against bacterial phytoene desaturases were used but only one against the protein from *Rhodobacter* showed cross reactivity (Fig. 2). The polyclonal antiserum differentially recognized a protein band of 63 kDa in the Western blot. This molecular weight corresponds well to a calculated molecular weight of 66 kDa for the *Neurospora* phytoene desaturase [9]. When equal amounts of dark- and light-grown mycelia were applied, a much stronger immunoreaction of the phytoene desaturase band in the latter was visible. There is also a non-specific protein band at 45 kDa. It developed an immunoreaction of comparable intensity and can therefore be regarded as an internal marker for equal amounts of total protein applied to each lane.

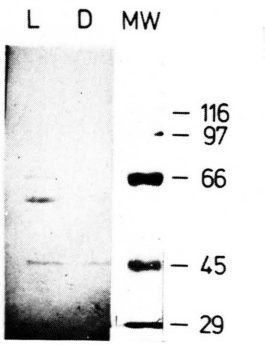


Fig. 2. Immunodetection of phytoene desaturase from light- and dark-grown *N. crassa* YLO on SDS polyacrylamide gels. L = extract from illuminated culture, D = from dark culture, MW = molecular weight markers.

## Discussion

Carotenogenesis in *Neurospora* is light-dependent [1]. As recently shown, regulation of this pathway is under transcriptional control. In case of two enzymes involved, phytoene desaturase encoded by the al-1 gene [9] and GGPP synthase encoded by the al-3 gene [10] an increase of the specific m-RNA was observed as an influence of light. The regulatory mechanism implies photo-induced changes in the amount and activities of carotenogenic enzymes.

For the first time *in vitro* formation of desaturated carotenes including cyclic γ-carotene could be observed with preparations from the *Neurospora* mutant YLO (Table II). The overall increase in the formation of total carotenes, phytoene, ζ-carotene, lycopene and γ-carotene was about 4- to 5-fold higher in light- *versus* dark-grown material. The contributions of individual enzymes to this enhanced synthesis was investigated by measuring and comparing their activity in appropriate mutants. Illumination of cultures stimulated the activities of phytoene desaturase 3-fold, GGPP synthase 2.3-fold, and phytoene synthase 1.4-fold. In contrast, FPP synthase and lycopene cyclase were unaffected by light treatment.

The extensive *in vitro* studies covering the individual reactions from DMAPP to γ-carotene give

a detailed picture on how carotenoid biosynthesis is enhanced in the light at the enzyme level (Table III). The results demonstrate conclusively a coordinated regulation of carotene synthesis. It involves a cascade mechanism in which the activities of three subsequent enzymes of the pathway from GGPP to lycopene are increased in the light. For one enzyme, phytoene desaturase, we could demonstrate by Western blot analysis that higher activities in the light correspond to a higher level of this specific protein (Fig. 2). The involvement of phytoene synthase in light regulation of carotenogenesis implies a possible translational regulation of the al-2 gene concurrent to the regulation of al-1 and al-3.

*In vitro* analysis of carotenogenic activities of other fungi with a light-regulated carotenoid biosynthetic pathway has been carried out with *Gibberella fujikuroi* [13] and *Aspergillus giganteus* [16]. In the *Gibberella* wild type, stimulation of carotenoid biosynthesis in the light mainly occurred at the level of phytoene synthesis. *Aspergillus* produced only negligible amounts of carotenoids in the dark. The enzymatic studies showed that phytoene synthase, phytoene desaturase as well as lycopene cyclase are totally absent in dark-grown cultures. A common general mechanism of photoregulation at the molecular level may be assumed. However, the consequences of photoregulation on enzyme activities and amounts of carotenoids formed are quite diverse [2]. Further detailed investigations on other photoinducible pathways in different fungi both at the molecular and enzymatic level may help our basic understanding of the whole sequence of events leading to photocontrol of a biosynthetic pathway.

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