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C_{35}-apocarotenoids in the yellow mutant *Neurospora crassa* YLO

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ABSTRACT

The *Neurospora crassa* mutant YLO exhibits a yellow phenotype instead of the red-orange pigmentation of the wild type. Recently, it was shown that the mutant YLO is defective in a specific aldehyde dehydrogenase which catalyses the last step of carotenogenesis to the formation of neurosporaxanthin [Estrada, A.F., Youssar, L., Scherzinger, D., Al-Babili, S., Avalos, J., 2008. The *ylo*-1 gene encodes an aldehyde dehydrogenase responsible for the last reaction in the Neurospora carotenoid pathway. Mol. Microbiol. 69, 1207–1220]. Since different carotenoid compositions between wild type and YLO have been reported in earlier publications, the carotenoids of YLO were analyzed and unknown carotenoids identified. Fractionation of carotenoid extracts from YLO revealed in the less polar fraction two major carotenoids of low polarity which were found only in trace amounts in the wild type. Both carotenoids could be hydrolyzed with KOH to more polar products indicating the presence of fatty acid esters. The fatty acid moiety was identified as myristic acid by gas chromatography. Optical and mass spectra as well as co-chromatography with a synthesized authentic standard identified the free alcohols as 4′-apolycopene-4′-ol and 4′-apo-γ-carotene-4′-ol which assigns the dominating carotenoids in the YLO mutant as 4′-apolycopene-4′-myristate and 4′-apo-γ-carotene-4′-myristate. We can attribute the accumulation of these two carotenoids in YLO to the substantial mutation of the neurosporaxanthin-forming aldehyde dehydrogenase. However, the aldehyde intermediates 4′-apo-γ-carotene-4′-al and 4′-apo-lycopene-4′-al do not accumulate substantially but are reduced instead to the corresponding alcohols, 4′-apolycopene-4′-ol and 4′-apo-γ-carotene-4′-ol, and both further esterified with mainly myristic acid yielding 4′-apolycopene-4′-myristate and 4′-apo-γ-carotene-4′-myristate.

1. Introduction

Carotenoids are essential protective pigments found in photoautotrophic organisms as well as in some heterotrophically growing bacteria. In fungi, carotenogenic species are present in all classes making their synthesis widespread in nature (Goodwin, 1980). In several species carotenoid biosynthesis is up-regulated by light (Rau and Schrott, 1987). This can be taken as an indication that carotenoids function as light-protecting pigments in these organisms. The predominating carotenoids in fungi are the bicyclic β-carotene, monocyclic γ-carotene and torulene as well as their hydroxy and keto derivatives (Sandmann and Misawa, 2002). Less common carotenoids can be found in Ascomycotina in the orders Spheriales and Pezzizales. They include plectaniaxanthin (3′,4′-didehydro-1′,2′-dihydro-β,ψ-carotene-1′,2′-diol) and its fatty acid ester from *Plectania coccinea* together with 2′-dehydroplectaniaxanthin in which the 2′-hydroxy group is replaced by a keto group (Arpin and Liaaen-Jensen, 1967a). Another carotenoid restricted to species of the Pezizales is aleuriaxanthin (1′,16′-dihydro-β,ψ-carotene-2′-ol) with a γ-carotene related structure and an additional 2′-hydroxy group and a 16′-double-bond (Arpin et al., 1973; Liaaen-Jensen, 1965). This compound was found in *Aleuria aurantia* and *Scutellinia umbrarum* (Schrantz and Lemoine, 1995). Phillipsiaxanthin (1′,1′-dihydroxy-3,4,3′,4′-tetradehydro-1,2,1′,1′-tetrahydro-ψ,ψ-carotene-2,2′-dione) the acyclic carotenoid from *Phillipsia carminea* (Arpin and Liaaen-Jensen, 1967a) is structurally related to 2′-dehydroplectaniaxanthin with 1-hydroxy and 2-keto groups at both sides of the acyclic molecule. Torularhodin (3′,4′-didehydro-β,ψ-carotene-16′-oic acid), an oxidation product of torulene with a 16′ carboxylic group was also reported for *Cookeina sulcipes* (Arpin and Liaaen-Jensen, 1967b) and *Pyronema confluens* (Goodwin, 1980).

In *Neurospora crassa*, the carotenoids are derived from either lycopene or from 3,4-didehydrolycopene with an additional double bond. The whole desaturation sequence to both carotenes is catalyzed by a single enzyme (Hausmann and Sandmann, 2000) which is the product of the *al-1* gene (Schmidhauser et al., 1990). Lycopene and 3, 4-didehydrolycopene are cyclized to γ- and ß-carotene or torulene, respectively, by a combined phytoene synthase/

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lycopene cyclase Al-2 (Schmidhauser et al., 1994; Sandmann et al., 2006). Finally, torulene can by cleaved by a carotenoid oxidase (Cao-2) at the 3,4,-double bond to C_{35} ß-apo-4′-carotenal (Saelices et al., 2007). In a final step, this aldehyde is oxidized to neurosporaxanthin (4′-apo-β,ψ-carotene-4′-oic acid) (Aasen and Jensen, 1965) by a recently identified aldehyde oxidase which is encoded by the gene *ylo-1* (Estrada et al, 2008). The whole carotenoid biosynthetic pathway in *N. crassa* is strongly light controlled (Rau and Schrott, 1987) due to the regulation of the *al* genes at the transcriptional level (Li and Schmidhauser, 1995; Sandmann and Misawa, 2002).

The carotenoid composition of the YLO mutants which is defective in *ylo-1* (Estrada et al., 2008) is still obscure. It was reported that neurosporaxanthin is absent in mutant YLO (Goldie and Subden, 1973; Estrada et al, 2008). Instead, other unknown polar carotenoids were found including those with a neurosporaxanthin/lycopene-related spectrum (Sandmann, 1993; Estrada et al., 2008). Therefore, we present here an analytical investigation of the carotenoids which accumulate in the yellow *N. crassa* mutant YLO. In the present work, it was possible to identify novel C_{35}-apocarotenoids which are responsible for the yellow phenotype of YLO.

2. Results and discussion

The *N. crassa* pigment mutant YLO shows a yellow phenotype. In contrast the wild type strain (WT) has a red-orange colour. The different pigmentation is indicated by the chromaticity b^*/a^* ratio which describes the degree of yellow colour (Arias et al., 2000). It is significantly higher for YLO than for WT (Table 1 Panel A). The mycelia of both strains were solubilised in Triton and the spectra recorded. For the WT the main maximum was at 478 nm (Fig. 1A). For YLO it was 14 nm yellow-shifted (Fig. 1B). However, when methanol extracts were prepared from WT and YLO, they exhibited a similar spectrum with maxima around 460 nm (data not shown).

Methanol extracts from both strains were separated on a silica column into a high and lower polar fraction. Both were analyzed by HPLC. In the polar fraction of the WT only all-trans neurosporaxanthin (peak 1) together with three *cis* isomers (peaks 1′, 1″, 1‴) were found (Fig. 2, trace A). Due to the enrichment on the silica column, small amounts of neurosporaxanthin were also found in the mutant YLO (Fig. 2, trace B). Two of the *cis* isomers were also present in the standard obtained by hydrolysis of ß-apo-4′-carotenoic acid ethylester (Fig. 2, trace C; spectrum in Fig. 1E). In the less polar fractions from WT and YLO, most of the carotenes of the pathway leading to β-carotene and β-zeacarotene were found. They include phytoene (peak 13), neurosporene (peak 5), lycopene (peak 4) and γ–carotene (peak 6) together with 3,4-dehydrolycopene (peak 3), a precursor of neurosporaxanthin (Fig. 4). In YLO, two prominent peaks 9 and 11 (Fig. 3B) with absorbance maxima 445, 470, 502 and 436, 460, 491 nm, respectively (Fig. 1C and D), appeared. In addition, polar compound 12 with a spectrum very similar to compound 11 were present. These carotenoids are completely unrelated to carotenoids from fungi and other organisms (Britton et al., 2004). In WT only trace amounts of peak 9 were detected and compound 2 which exhibited absorbance maxima at 425, 455 and 483 nm which could not be assigned due to its low amounts.

Quantitation of the carotenoids of WT and YLO indicate that in this fungus carotenoid biosynthesis is not tightly regulated in a way that mainly one or two end products accumulate and the intermediate pools are very low or below detection. This is often the case in other species (Sandmann and Misawa, 2002). The highest concentrations were found for phytoene, the carotene in the pathway and γ-carotene which is a precursor for β-carotene and neurosporaxanthin (Fig. 4). The main quantitative differences

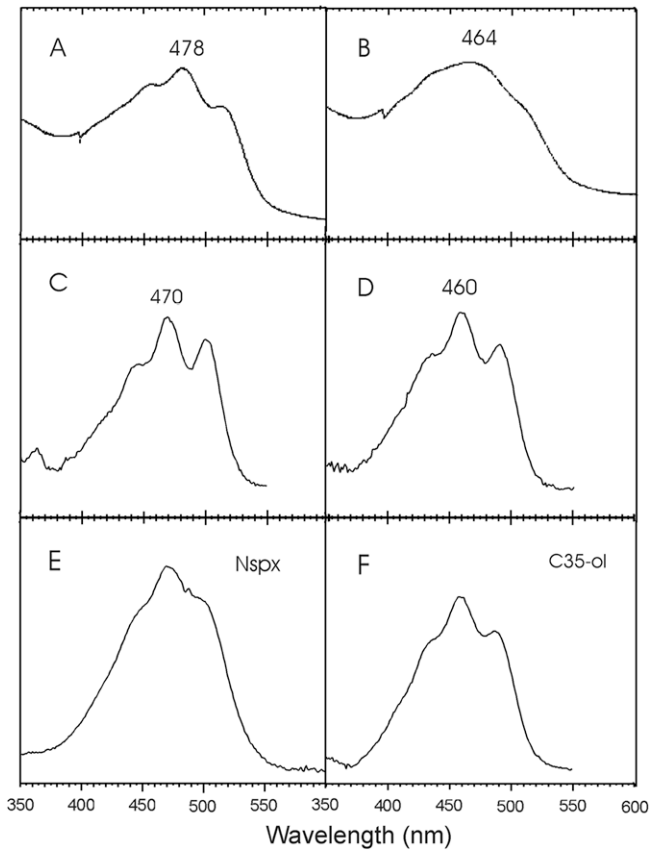

Fig. 1. Optical absorbance spectra of Triton X-100 solubilisates from *Neurospora crasse* wild type (A) and mutant YLO (B), carotenoids P470 (C; Rt 38.3 min. in Fig. 3B), P460 (D, Rt 50.4 min. in Fig. 2C) and the reference compounds neurosporaxanthin (Nspx, E) and 4′-apo-γ-carotene-4′-ol (C35-ol, F).

between WT and YLO carotenoid composition is the low neurosporaxanthin content in YLO (Table 1 Panel B). Instead, compounds 9, 10 and 11 accumulated in YLO. Total carotenoid content in YLO is about 1.6-fold higher than in WT. This is attributed to higher accumulation of phytoene and β-carotene in YLO. In addition, the much lower amounts of neurosporaxanthin in YLO are overcompensated by compounds 9–11 which are more than 2-fold

Table 1
Color reading (A) and carotenoid composition (μg/g dw) (B) of *Neursopora crassa* and the yellow mutant YLO

	Wild type	Mutant
Panel A		
Relative chromaticity b^*/a^* ratio	1.55 ± 0.05	2.01 ± 0.16
Panel B		
Neurosporaxanthin	156.9 ± 38.0 (12%)	20.8 ± 8.6 (1%)
Compound 10	0 (0%)	67.4 ± 9.3 (3%)
Compound 2	85.0 ± 17.5 (6%)	0 (%)
3,4-Didehydrolycopene	71.9 ± 7.2 (5%)	112.7 ± 15.4 (5%)
Lycopene	132.3 ± 20.9 (10%)	67.4 ± 8.7 (3%)
Neurosporene	110.4 ± 20.1 (8%)	78.3 ± 10.6 (4%)
γ-Carotene	334.9 ± 95.8 (24%)	383.9 ± 43.4 (17%)
β-Zeacarotene	23.7 ± 7.8 (2%)	53.4 ± 8.2 (2%)
β-Carotene	54.0 ± 13.1 (4%)	272.6 ± 41.9 (12%)
Compound 9	25.0 ± 2.9 (2%)	132.8 ± 25.1 (6%)
Compound 11	7.4 ± 2.4 (1%)	249.1 ± 39.5 (11%)
Phytoene	362.5 ± 44.2 (27%)	804.1 ± 91.3 (36%)
Total carotenoids	1364.0	2242.5

Means from 5 samples ± SD.

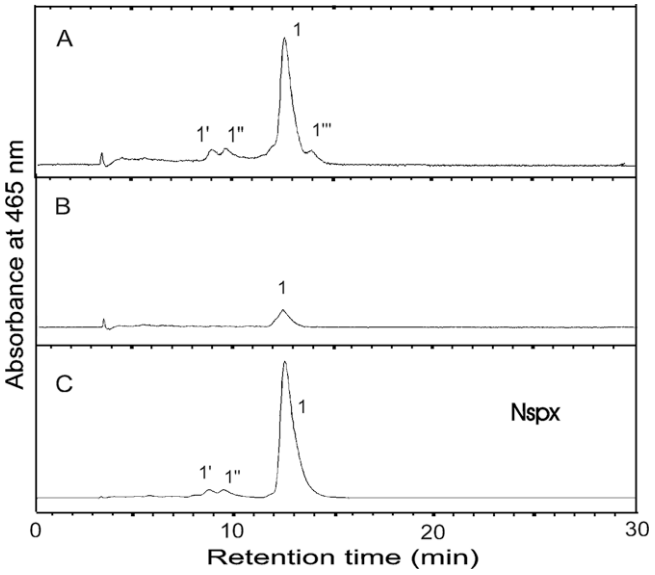

Fig. 2. HPLC separation on a C30-column of the polar fraction absorbed on a silica column; (A). from wild type *Neurospora crassa* and (B). from mutant YLO. (C). A neurosporaxanthin standard.

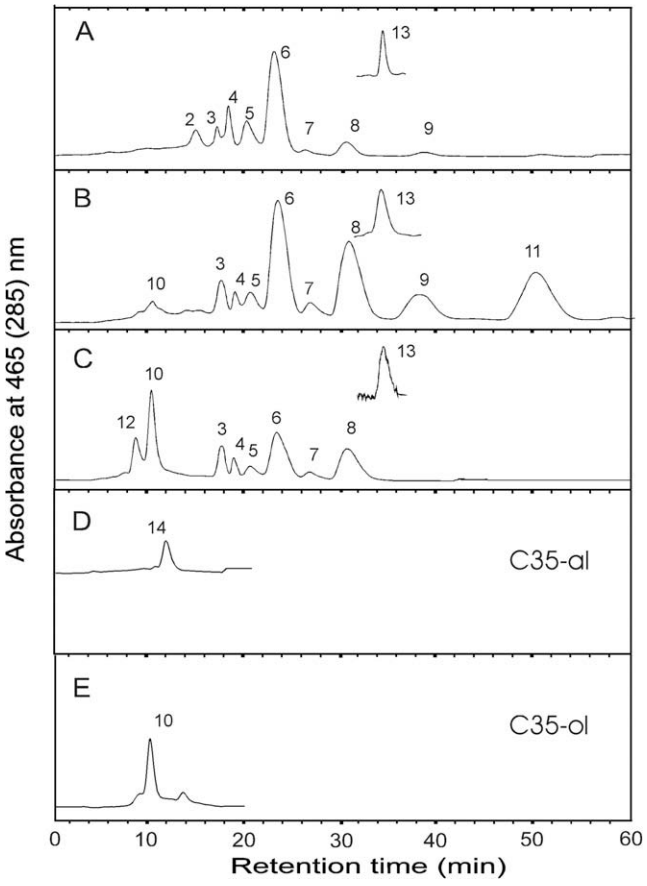

Fig. 3. HPLC separation on a C18-column of the less polar carotenoid fraction from *Neurospora crasse* wild type (A) and mutant YLO (B). Trace C corresponds to a hydrolysed extract from YLO, D shows the reference compound 4′-apo-γ-carotene-4′-al and E 4′-apo-γ-carotene-4′-ol. The region with a peak around 34 min recorded at 285 nm which indicates phytoene was inserted into traces A to C.

increased. In *Fusarium fujikuroi*, a *N. crassa* related fungus with neurosporaxanthin biosynthesis (Avalos and Cerda-Oledo, 1986), a mutation in the dioxygenase gene preventing the formation of C_{35}-apocarotenoids including neurosporaxanthin results in an almost 2-fold increase of carotenoid synthesis (Thewes et al., 2005). A similar end-product regulation is typical for β-carotene biosynthesis in *Phycomyces blakesleeanus* (Murillo and Cerdá-Olmedo, 1976). The presence of low concentrations of neursoporaxanthin in YLO indicates that the mutation in the aldehyde dehydrogenase results in a modified enzyme with a residual catalytic activity.

Upon hydrolysis of the carotenoid extract from YLO, compounds 9 and 11 disappear and two other more polar carotenoid nos. 12 and 10 are found instead by HPLC (Fig. 3C). They exhibit the same spectra as nos. 9 and 11, respectively (Fig. 1C and D). This feature is typical for hydrolysis of carotenoid fatty acid esters. By gas chromatography, myristic acid was identified as the major fatty acid after hydrolysis of isolated compounds 9 and 11. This fatty acid is efficiently synthesized in *N. crassa* (Mikolajczyk and Brody, 1990). Minor fatty acids bound to the C_{35} carotenoid alcohols are 16:0, 18:0, 18:1 and 18:3. Mass spectra of the free carotenoid alcohols 10 and 12 gave a mass of 484 in each case (Table 2). Due to the spectral properties listed in Table 2 and their relationship to neurosporaxanthin biosynthesis, compound 9 was assigned as 4′-apolycopene-4′-myristate and compound 11 as 4′-apo-γ-carotene-4′-myristate. Although both carotenoids exhibit the same mass, the absorbance spectra indicate a different chromophore of 11 acyclic double bonds for 4′-apolycopene-4′-myristate and its alcohol with maxima at 445, 470 and 502 nm or of 10 acyclic double bond in conjugation with the C-5,6 double bond in the β-ionone ring with maxima of 436, 460 and 491 nm for 4′-apo-γ-carotene-4′-myristate and its alcohol (Takaichi and Shimada, 1992). The corresponding 4′-apo-γ-carotene-4′-ol was synthesized from ß-apo-4′-carotenoic acid ethylester by reduction with $LiAlH_4$. It had the same properties and co-chromatographed with compound 11 (Fig. 1F, Fig. 3E). The 4′-apo-γ-carotene-4′-al obtained by oxidation of the alcohol was used as a standard for chromatography (Fig. 3D) to demonstrate that 4′-apo-γ-carotene-4′ is absent from *N. crassa* WT and the YLO mutant. Both C_{35}-carotenoid fatty acid esters have not been found before (Britton et al., 2004). The only other modified C_{35} carotenoid was found in the marine *Fusarium* strain T-1 (Sakaki et al., 2002). It accumulates a neurosporaxanthin β-D-glucopyranoside which is accompanied by neurosporaxanthin.

In YLO, most of the neurosporaxanthin is replaced by 4′-apolycopene-4′-myristate and 4′-apo-γ-carotene-4′-myristate (Table 1B). Although YLO shows a yellow coloration in contrast to the wild type (Table 1A), the spectra from methanolic cell extracts looked very similar and composition of yellow, orange and red carotenoids in both strains are not substantially different (Table 1B). The reason for the yellow colour of YLO should be due to the properties of the accumulating 4′-apolycopene-4′-myristate and 4′-apo-γ-carotene-4′-myristate. During separation by TLC, both esters looked yellow on the silica plate. We assume that the yellow colour of YLO mycelium and the Triton extracts can be attributed to a matrix interaction of the fatty acid moiety of 4′-apolycopene-4′-myristate and 4′-apo-γ-carotene-4′-myristate. There is an example of *P. blakesleeanus* mutants with a colour shift (de la Concha and Murillo, 1984). As in the wild type, the mutant contains β-carotene as the only carotenoid but it forms a protein aggregate which is responsible for the change in pigmentation.

In conclusion, the mutation in the aldehyde dehydrogenase in the *N. crassa* YLO mutant (Estrada et al., 2008) strongly decreases the conversion of 4′-apo-γ-carotene-4′-al to neurosporaxanthin and the oxidation of 4′-apolycopene-4′-al. These aldehydes are not accumulated but reduced to their corresponding alcohols. The absence of 4′-apo-γ-carotene-4′-myristate in the WT can be

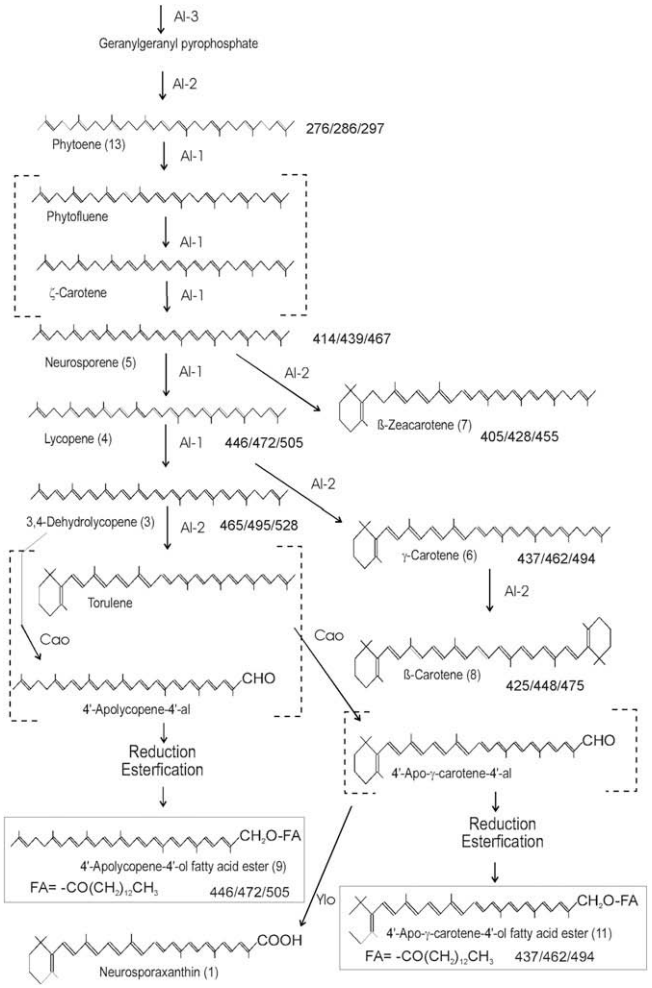

Fig. 4. Biosynthesis pathway for the formation of neurosporaxanthin, 4′-apo-γ-carotene-4′-myristate and 4′-apo-lycopene-4′-myristate in *Neurospora crassa*. Known gene products catalyzing the individual reactions are indicated. Preferentially accumulating carotenoids in YLO are boxed and the conversion of 4′-apo-γ-carotene-4′-al to neurosporaxanthin is partially blocked in this mutant. Carotenoids in brackets are postulated intermediates. Numbers indicate the peaks in Figs. 2 and 3.

explained by the successful competition of aldehyde oxidase with the still unknown reductase for 4′-apo-γ-carotene-4′-al. In YLO, the alcohols are further esterified mainly with myristic acid yielding 4′-apolycopene-4′-myristate and 4- ′apo-γ-carotene-4′-myristate. In addition trace amounts of other fatty acid esters are formed. These compounds are minor in *N. crassa* WT. The partial disruption of neurosporaxanthin synthesis and accumulation of C_{35} diapocarotenoic fatty acid esters exerts a positive regulatory effect on total carotenoid synthesis and the metabolite flow especially into the β-carotene branch of the *N. crassa* carotenoid biosynthesis pathway.

3. Experimental

3.1. Organism and cultivation

N. crassa wild type strain 74-OR23-1A and its yellow mutant Y3059y (strain FSG4100) were cultivated for 4 days in Vogel's minimal medium (Davis and de Serres, 1970) on a shaker at 25 °C gassed with a stream of air (5 l/min.). Inoculation was with freshly produced spores.

3.2. Color determination of mycelia and spores

The reflectance spectra from freeze-dried and powdered mycelia in an optical cuvette were obtained using a portable HunterLab MiniScan XE Plus spectrophotometer equipped with a built-in xenon flash light source. Ratios of chromaticity coordinates b^* (blue to yellow scale) vs. a^* (green to red scale) were calculated (Arias et al., 2000). Absorbance spectra from mycelia were recorded in a photometer after solubilization of the mycelium in 1% Triton X-100 in 0.2 M phosphate buffer pH 7.5 by treatment with a potter homogenizer.

3.3. Carotenoid analysis

Carotenoids were extracted from 30 mg of freeze dried mycelium either with methanol or with methanol containing 6% KOH by heating for 15 min at 60 °C. After filtration, the residue was re-extracted with acetone. Partitioning of the combined extracts against 10% ether in petrol concentrated the carotenoids in the upper phase. These extracts were used for purification by TLC or fractionation prior to HPLC analysis. All extracts were fractionated on silica columns by dissolving the carotenoids in 10% acetone in petrol and passing through the column. Only neurosporaxanthin was retained under these conditions. It was eluted with acetone and then separated and quantitated by HPLC on a non-end capped polymeric 3 μm C30 column (YMC Wilmington NC, USA) eluted with methanol/methyl-*tert*-butyl ether/water (56:40:4, by volume) (Sander et al., 1994). The HPLC system for the other carotenoids involved a Nucleosil C18 3 μ column with acetonitrile/methanol/2-propanol (85:10:5, by volume) as the mobile phase for isocratic elution (Steiger et al., 1999). Flow rates were 1 ml/min and the column temperature 26 °C. Detection was with a Kontron DAD 440 photodiode array detector with on-line registration of the spectra. For mass spectroscopy, non-saponified carotenoids were isolated by TLC on silica gel developed with toluene/petrol/acetone (15:85:5, by volume). The carotenoid bands were scraped of eluted and saponified. Then, they were further purified in a

Table 2
Characteristics and identification of the apocarotenoids from *Neurospora crassa*

Compound[a]	Absorption maxima (nm)[b]	Molecular mass (*m*/*z*)	Major fatty acid[c]	Carotenoid
#9	445, 470, 502	nd	14:0	4′-Apolycopene-4′-Myristate
#11	436, 460, 491	nd	14:0	4′-Apo-γ-carotene-4′-myristate
#12[d]	445, 470, 502	484	None	4′-Apolycopene-4′-ol
#10[e]	436, 460, 492	484	None	4′-Apo-γ-carotene-4′-ol

[a] Peak nubering according to Fig. 2.
[b] In the HPLC mobile phase acetonitrile/methanol/2-propanol (85:10:5).
[c] Minor fatty acids identified were 16:0, 18:0, 18:1, 18:3.
[d] Hydrolysis product of peak 9.
[e] Hydrolysis product of peak 11. nd not determined.

second step by HPLC. Reference carotenoid 4′-apo-γ-carotene-4′-ol was obtained by reducing ß-apo-4′-carotenoic acid ethylester with $LiAlH_4$ and oxidized further to 4′-apo-γ-carotene-4′-al with chloranil (Eugster, 1995). Both products were purified on silica plates developed with toluene/ethylacetate/methanol (75:20:5, by volume). Neurosporaxanthin was obtained by ester hydrolysis with 10% KOH in methanol at 60 °C for 30 min. and purification from the residual ester on a silica column as described above. Carotenoids from wild type and YLO were quantitated from five individual cultures. For all carotenes, authentic standards generated by combinatorial biosynthesis in *Escherichia coli* were available (Sandmann, 2002).

3.4. Mass and fatty acid determination

The masses of the carotenoid products were determined by MALDI-TOF. The α-cyan-4-hydroycinnamic acid (CHCA) matrix solution was prepared in acetone at 1 to 10 mg/ml. Carotenoid or crude extract (1 μl) was mixed with an equal volume of matrix and 0.5 μl spotted onto the target plate. MALDI-TOF spectra were acquired with a Bruker MALDI-TOF Reflex III (Coventry, UK) operated in the positive reflectron mode. A nitrogen laser emitting at 337 nm was used to generate ions. A source voltage of 25 KV was used, 20.7 KV extraction voltage and no ion suppression was employed. Data were acquired with 15 shots/scan, 3 scans/spectrum. The mass spectrometer was calibrated with authentic carotenoid standards.

Analysis of fatty acid methyl esters (FAMEs) was performed using a Varian (Wlaton-on-Thames, Surrey, UK) CP3380 GC with a CP8410 autosampler. 1 μl injections were made in a splitless mode. The injector temperature used was 180 C. Separations were performed on a CP-Sil 88 column designed for FAME analysis and supplied by Varian, placed in column oven programmed for 140 C initially ramping to 240 over 27 min increasing at a rate of 4C/min. These conditions were held until 35 min. The FID detector temperature was maintained at 26 °C.

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