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RESEARCH ARTICLE

Carotenoids and carotenogenic genes in *Podospora anserina*: engineering of the carotenoid composition extends the life span of the mycelium

Ingmar Strobel · Jürgen Breitenbach · Christian Q. Scheckhuber · Heinz D. Osiewacz · Gerhard Sandmann

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Abstract Carotenoids have been identified in the fungus *Podospora anserina* and a parallel pathway to neurosporene and β-carotene was established. Three genes for the β-carotene branch have been cloned and their function elucidated. They correspond to the *al-1*, *al-2* and *al-3* genes from *Neurospora crassa*. They were individually and in combinations over-expressed in *P. anserina* in order to modify the carotenoid composition qualitatively and quantitatively. In the resulting transformants, carotenoid synthesis was up to eightfold increased and several intermediates of the pathway together with special cyclic carotenoids, β-zeacarotene and 7,8-dihydro-β-carotene, accumulated. All transformants with an over-expressed *al-2* gene (encoding a phytoene synthase and a lycopene cyclase) displayed up to 31% prolonged life span.

Keywords Ageing protection · *al* Genes · Carotenoid biosynthesis · Metabolic engineering · Neurosporaxanthin

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J. Breitenbach · G. Sandmann (✉)
Biosynthesis Group, Department of Biological Sciences, Institute of Molecular Biosciences, J. W. Goethe Universität, P.O. Box 111932, 60054 Frankfurt, Germany
e-mail: sandmann@bio.uni-frankfurt.de

I. Strobel · C. Q. Scheckhuber · H. D. Osiewacz
Molecular Developmental Biology, Department of Biological Sciences and Cluster of Excellence Macromolecular Complexes, Institute of Molecular Biosciences, J. W. Goethe University, Max-von-Laue-Str. 9, 60438 Frankfurt, Germany
e-mail: Ingmar.Strobel@gmx.de

Introduction

Carotenoids are antioxidative pigments. They have the potential to inactivate radicals, quench singlet oxygen and dissipate the energy from excited photo sensitizers as heat (Britton 1995). For photosynthetic organisms, the presence and synthesis of carotenoids is essential since they act as antioxidants (Siefermann-Harms 1987). Carotenoids are also abundant in heterotrophic organisms including fungi (Sandmann and Misawa 2002). There, they protect against oxidative stress and against photoreactions sensitized by porphyrins and riboflavins. For example, *Ustilago violacea* has a high cytochrome *c* content and different strains may additionally contain or lack carotenoids. Treatment with visible or UV radiation revealed that carotenogenic strains are more resistant against photodestruction (Will et al. 1984). Similar protection by carotenoids was observed also for *Neurospora crassa* (Blanc et al. 1976). In several fungi, carotenogenesis is photoinduced as part of their antioxidative system (Rau and Schrott 1987).

Fungal carotenogenesis and carotenoid composition is diverse. In Zygomycota, the typical carotenoid is β-carotene (Goodwin 1980) whereas in Ascomycota and Basidiomycota carotenoid composition is more diverse ranging from torularhodin (3′,4′-didehydro-β,ψ-carotene-16′-oic acid), hydroxytorulene (3′,4′-didehydro-β,ψ-carotene-16′-ol) and canthaxanthin (β,β-carotene-4,4′-dione) to astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) (Buzzini et al. 2007; Goodwin 1980; Herz et al. 2007).

In Ascomycetes, most carotenoids are derived from 3,4-dehydrolycopene and its monocyclic derivative torulene such as the acyclic phillipsiaxanthin (1′,1′-dihydroxy-3,4,3′,4′-tetradehydro-1,2,1′,1′-tetrahydro-ψ,ψ-carotene-2,2′-dione) in *Phillipsia carminea* (Arpin and Liaaen-Jensen 1967a), cyclic plectaniaxanthin (3′,4′-didehydro-1′,

2′-dihydro-β,ψ-carotene-1′,2′-diol) in *Plectania coccinea* (Arpin and Liaaen-Jensen 1967a), aleuriaxanthin (1′,16′-dihydro-β,ψ-carotene-2′-ol) (Arpin et al. 1973) in *Aleuria aurantia* and *Scutellinia umbrarum* (Schrantz and Lemoine 1995), torularhodin (3′,4′-didehydro-β,ψ-carotene-16′-oic acid) in *Cookeina sulcipes* (Arpin and Liaaen-Jensen 1967b) and *Pyronema confluens* (Goodwin 1980) or neurosporaxanthin (4′-apo-β,ψ-carotene-4′-oic acid) in *N. crassa* (Aasen and Jensen 1965). The latter carotenoid is derived from torulene by oxidative cleavage at C-5′ and further oxidation.

Neurospora crassa is one of the few fungi from which all genes of the whole carotenogenic pathway have been cloned and functionally identified. The pathway is catalyzed by the product of the *al-2* gene, which encodes a phytoene synthase and a lycopene cyclase (Schmidhauser et al. 1994; Sandmann et al. 2006), and of the *al-1* gene encoding a phytoene desaturase for the synthesis of lycopene and 3,4-dehydrolycopene (Schmidhauser et al. 1990; Hausmann and Sandmann 2000). Cleavage of torulene and oxidation to neurosporaxanthin is mediated by the genes of a carotenoid oxidase *cao-2* (Saelices et al. 2007) and an aldehyde oxidase *ylo-1* (Estrada et al. 2008). Another fungus with a carotenogenic biosynthesis pathway leading to neurosporaxanthin (Avalos et al. 1988) with similar genes involved is *Fusarium fujikuroi* (Thewes et al. 2005). However, it is open whether other related fungi among the Spheriales are able to synthesize neurosporaxanthin or other carotenoids and what the function of carotenoids is in these fungi.

In the present investigation, we analyzed the carotenoids of *Podospora anserina*, an intensively studied model system in ageing research. In this filamentous ascomycete, ageing and life span is controlled by a complex network of molecular pathways in which mitochondria plays a paramount role (for reviews see: Lorin et al. 2006; Osiewacz 2002; Scheckhuber and Osiewacz 2008). According to the mitochondrial free radical theory of ageing (Harman 1956; Harman 1972, 1992), the formation of reactive oxygen species (ROS) at the respiratory chain and their potential to damage nucleic acids, proteins and lipids results in cellular dysfunction and degeneration of biological systems. Since it is known that carotenoids are part of a cellular defence system against ROS, we set out to elucidate the ability of these compounds to influence the ageing process of *P. anserina*. Here, we report the cloning of three genes for enzymes involved in the early stage of carotenogenesis and their functional characterization. Over-expression of these genes resulted in qualitative and quantitative modifications of the carotenoid composition. Transgenic strains exhibited an extended life span supporting the beneficial role of carotenoids during the life cycle of *P. anserina* cultures.

Materials and methods

Strains and cultivation

Over-expression strains *PaAl1*_Ex, *PaAl2*_Ex and *PaAl3*_Ex (this study) were derived from wild type strain "s" (Esser 1974). If not noted otherwise, the *P. anserina* isolates used in this study were grown on cornmeal agar (Esser 1974) or synthetic medium (PASM + 1% glucose) in an incubation chamber at 27°C. Composition of PASM + 1% glucose (per litre): 10 g glucose, 0.5 g KH_2PO_4, 0.6 g K_2HPO_4, 1 g urea, 2.5 µg biotin, 50 µg thiamine, 5 mg citric acid × H_2O, 5 mg $ZnSO_4 \times 7H_2O$, 1 mg $Fe(NH_4)_2(SO_4)_2 \times 6H_2O$, 0.25 mg $CuSO_4 \times 5H_2O$, 0.05 mg $MnSO_4 \times H_2O$, 0.05 mg H_3BO_3, 0.05 mg $Na_2MoO_4 \times 2H_2O$.

Identification of *PaAl-1*, *PaAl-2* and *PaAl-3* in the genomic *P. anserina* sequence

The published amino acid sequences of *N. crassa* genes *al-1* (UniProt Accession P21334), *al-2* (UniProt Accession P37295) and *al-3* (UniProt Accession P24322) were used to search the genomic sequence of *P. anserina* [Espagne et al. 2008), published at http://www.genoscope.cns.fr/externe/English/Projets/Projet_GA/GA.html (*P. anserina* sequencing consortium)] for the corresponding homologs via the BlastX (protein sequence vs. nucleotide sequence) algorithm. The genomic sequence in this data base is derived from *P. anserina* wild type strain "S". Since all experiments in this study were performed with a different wild type strain, "s", the sequence of the cloned genes was verified by comparison with the published sequences. No differences of sequences were found. The accession numbers assigned by the *P. anserina* sequencing consortium are Pa_1_15010 (*PaAl-1*), Pa_1_15240 (*PaAl-2*) and Pa_2_610 (*PaAl-3*), respectively. Homology comparisons were performed using the BLOSUM62 algorithm implemented in "Clone Manager Suite 6" (Scientific and Educational Software, Cary, NC).

Cloning of carotenogenic genes and genetic manipulation of *P. anserina*

To overexpress *PaAl-1* in *P. anserina* wild type, the gene including the terminator region was amplified using primers *Bam*HI-Al1_f2 (5′-AAGGATCCATGGCGAATGAGAAGCCAAGG-3′) and XbaI-Al1_r2 (5′-AATCTAGATCGTAATCGAGTCCACCC-3′). The PCR fragment was cloned after digestion into the *Bam*HI/*Xba*I site of vector pExMthph containing an 0.56 kbp promoter fragment of the metallothionein gene (*PaMt1*) (Averbeck et al. 2001) and a resistance cassette for selection on hygromycin

B-containing medium. For the overexpression of *PaAl-2* in *P. anserina* wild type, the gene including the terminator region was amplified using primers *Bam*HI-Al2_f2 (5′-AAGGATCCATGGCTTACGATTATGCC-3′) and BcuI-Al2_r2 (5′-CCACTAGTTATGTGATATGTTGTGCC-3′). After digestion the PCR fragment was cloned into the *Bam*HI/*Bcu*I site of vector pExMthph.

To overexpress *PaAl-3* in *P. anserina* wild type, the gene including the terminator region was amplified using primers *Bam*HI-Al3_f2 (5′-AAGGATCCATGTCGTCACCAACATCG-3′) and *Hin*dIII-Al3_r2 (5′-CCCAAGCTTTTGGTGTTGCTGTCCACG-3′). After digestion the PCR fragment was cloned into the *Bam*HI/*Hin*dIII site of vector pExMthph. The constructs were termed pAl-1_Ex, pAl-2_Ex, pAl-3_Ex and verified to be error-free by sequencing the insertion fragments. The constructs used in this study were transformed into *P. anserina* protoplasts according to previously published protocols (Osiewacz et al. 1991; Stumpferl et al. 2004). 10^7 protoplasts were used in each transformation.

Southern blot analysis

Total DNA was isolated and digested with *Hin*dIII (MBI Fermentas) manufacturer's instructions. The resulting fragments were separated on 1% standard TAE agarose gels. DNA was transferred onto nylon membranes and hybridized with DIG-labelled probes for 16 h at 65°C in "High-SDS" hybridization buffer. For the detection of *PaAl-1*, the DIG-labelled 0.79 kbp *Bam*HI/*Kpn*I fragment of plasmid pAl-1_Ex was used. *PaAl-2* was detected by DIG-labelling the 0.74 kbp *Pst*I fragment of Plasmids pAl-2_Ex. For the detection of *PaAl-3*, the 0.86 kbp *Xho*I fragment of plasmid pAl-3_Ex was DIG-labelled. Southern blots were washed after hybridization twice for 5 min at RT in 2× SSC/0.5% SDS and twice for 15 min at 68°C in 0.1× SSC/0.1% SDS.

Functional complementation of genes in *Escherichia coli* and carotenoid analysis

The function of the three *al* genes from *P. anserina* was assessed by pathway complementation. Plasmids with the cDNAs of *al-1*, *al-2* and *al-3* were constructed in frame under the *lacZ* promoter by PCR amplification and ligation of the reaction products with T-overhanging ends into the *Xcm*I-digested vector pMON3820 (Borovkov and Rivkin 1997). These plasmids were co-transformed with other carotenogenic plasmids and the resulting carotenoids analyzed in the transformants *E. coli*/pMONal-1/pACCRT-EB, *E. coli*/pMONal-2/pACCRT-E, *E. coli*/pMONal-2/pACCRT-EBI, *E. coli*/pMONal-1/pACCRT-EB and *E. coli*/pMONal-1/pACCAR25ΔcrtE. Plasmid pACCRT-E contains a GGPP synthase gene, pACCRT-EB an additional phytoene synthase gene and pACCRT-EBI as a third gene that of a phytoene desaturase all on plasmid pACYC 184 (Misawa et al. 1995). Plasmid pACCAR25ΔcrtE carries all genes for the synthesis of zeaxanthin glucosides except the GGPP synthase gene *crtE* (Misawa et al. 1990).

For carotenoid analysis, pulverized freeze-dried mycelium or *E. coli* cells were first extracted with 6% KOH in methanol and then re-extracted with acetone for 15 min at 60°C each time. The carotenoids were partitioned into 30% ether in petrol. Two HPLC systems were used for carotenoid analysis. System I involved a non-end capped polymeric 3 µm C30 column (YMC Wilmington NC, USA) (Sander et al. 1994) and elution with methanol/methyl-*tert*-butyl ether/water (56:40:4, by volume) for 40 min followed by a change to methanol/methyl-*tert*-butyl ether/water (26:70:4, by volume) at a column temperature of 26°C (Breitenbach et al. 2001). System II involved a Hypersil HyPurity Elite C18 3μ column with acetonitrile/methanol/2-propanol (85:10:5, by volume) as the mobile phase for isocratic elution at a column temperature of 32°C (Ojima et al. 2006). The formation of neurosporaxanthin and its aldehyde was previously described (Sandmann et al. 2008). Authentic carotenoid standards for HPLC were generated by combinatorial biosynthesis in *E. coli* (Sandmann 2002).

Podospora anserina life span analysis

Ascospores from the wild type strain "s" and the transgenic strains were isolated and germinated on cornmeal agar supplemented with 60 mM ammonium acetate. After 3 days of incubation, a small piece cut from the juvenile mycelium was placed on into a race tube containing 50 ml PASM + 1% glucose. Growth was measured until the isolate stopped growing. Such cultures are defined as "dead". The elapsed time to this point was recorded as the life span of the corresponding isolate. The mean life span is defined as the time point when 50% of the analyzed cultures have died.

Results

Identification of the carotenoids in *P. anserina* and functional characterization of first genes of the carotenoid biosynthesis pathway

Upon carotenoid separation on a C30 HPLC column, three prominent peaks at 13 and 22 min with absorbance maxima of 445, 472 and 505 nm and at 20 min with absorbance maxima of 425, 448 and 475 nm were found (Fig. 1a). They all could be identified with authentic standards exhibiting the same retention times and spectra as neurosporaxanthin (4′-apo-β,ψ-carotene-4′-oic acid), its aldehyde

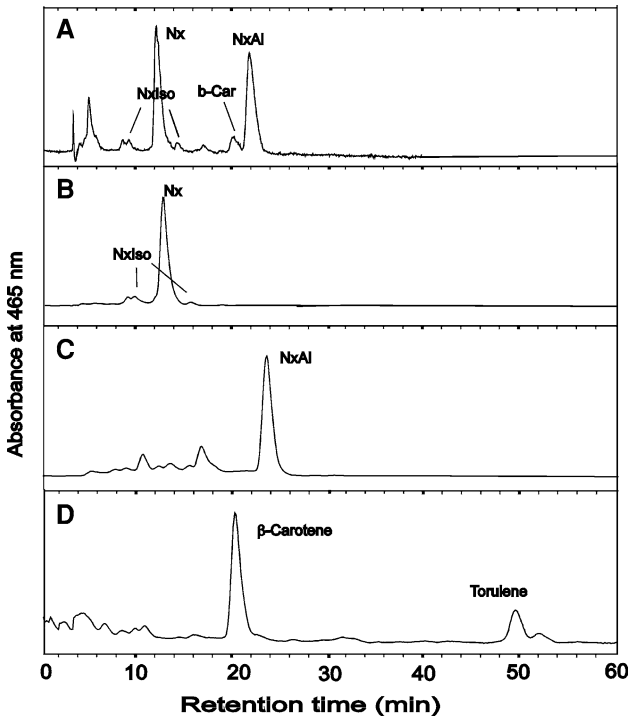

Fig. 1 Identification by HPLC of the carotenoids in extracts from *Podospora anserina* wild type (**a**) Authentic standards used as references were neurosporaxanthin (*Nx* **b**), 4′-apo-γ-carotene-4′-al (*Nxal* **c**) and β-carotene + torulene (*b-Car* **d**). Separation was on a C30 column eluted with methanol/methyl-*tert*-butyl ether/water. *Iso* indicates cis isomers

(4′-apo-γ-carotene-4′-al) and β-carotene (Fig. 1b–d). In addition, two minor peaks (NspxIso) around the main neurosporaxanthin peak could be assigned as its cis isomers. The three products of the branched carotenogenic pathway of *P. anserina* are boxed in Fig. 2, which shows the entire pathway in analogy to *N. crassa* (Estrada et al. 2008).

The putative carotenogenic genes of *P. anserina* (named *PaAl-1* to *PaAl-3* like the corresponding genes from *N. crassa*) were identified in the genomic *P. anserina* database (Espagne et al. 2008). As expected, the proteins encoded by these genes were highly conserved with the corresponding *N. crassa* enzymes. Sequence identity was 61% (Al-1) and 57% (Al-2, Al-3), respectively. After the cloning of *PaAl-1*, *PaAl-2* and *PaAl-3*, functional complementation analyses were performed in order to validate the proposed activities of the enzymes encoded by these genes (Fig. 2). In *E. coli* with a phytoene background, the gene *PaAl*-1 mediated the desaturation of this carotene to neurosporene in two steps and further on to lycopene and 3,4-dehydrolycopene in a total of five desaturations (Fig. 3a). Since fungi typically possess fusion genes for phytoene synthase and lycopene cyclase and *al-2* was identified as such a gene (Schmidhauser et al. 1994; Sandmann et al. 2006). When an *al-2* expression plasmid co-transformed in *E. coli* either with a plasmid responsible for the generation of geranylgeranyl pyrophosphate (Fig. 3b) or with a plasmid which mediates the synthesis of lycopene (Fig. 3c), formation of phytoene and cyclic β-carotene together with monocyclic γ-carotene, respectively, was observed. The function of *al-3* as a gene for a geranylgeranyl pyrophosphate synthase was demonstrated by co-transformation with a plasmid that contains all genes for the synthesis of zeaxanthin diglucoside except for the geranylgeranyl pyrophosphate synthase gene and is therefore unable to mediate the formation carotenoids (Fig. 3d). In the presence of *al-3*, zeaxanthin together with its mono- and diglucoside is synthesized.

Transgenic strains over-expressing genes of the carotenoid biosynthesis pathway

In order to alter the composition and the concentration of *P. anserina* carotenoids and study the resulting effects on the phenotype, we set out to create transgenic strains in which one or more *PaAl* genes are over-expressed. The constructs for *PaAl* over-expression (see "Materials and methods") were transformed into *P. anserina* wild type protoplasts. Putative transformants were selected on hygromycin B-containing medium. The complete integration of the expression cassettes into the genome of transformants by non homologous end-joining (i.e. direct ligation of strand ends independent of DNA homology) was verified via Southern blot analysis (Fig. 4a). This led to the identification of transgenic strains which contain a single copy of the corresponding expression cassette (PaAl-1_Ex T2, PaAl-2_Ex T7 and PaAl-3_Ex T2). In addition to creating mutants in which a single gene of the carotenoid biosynthesis is over-expressed, we constructed the two double mutants PaAl-1_Ex/PaAl-2_Ex and PaAl-2_Ex/PaAl-3_Ex by crossing the single mutants to each other and selecting progeny in which genetic recombination has occurred. The double mutants were also verified by Southern blot analysis (Fig. 4b).

The carotenoid composition of the various *P. anserina* mutants with engineered carotenogenic pathway was analyzed by HPLC (Fig. 5). Single transformants with over-expressed *PaAl-1* and *PaAl-3* genes, respectively, showed a very similar carotenoid pattern and additionally a minor torulene peak. In PaAl-1_Ex, a novel peak 485 appeared at 9 min. Its absorbance spectrum exhibited maxima at 455, 485 and 515 nm. By partitioning between ether and acidic or alkaline phases, it behaved like neurosporaxanthin indicating that this carotenoid is also an acid. We tentatively assigned it as 4′-apo-lycopene-4′-oat. The corresponding alcohol was recently found esterified with fatty acids in *N. crassa* (Sandmann et al. 2008). In contrast, PaAl-2_Ex with the over-expressed *PaAl-2* gene synthesized several additional carotenes. They include the acyclic phytoene

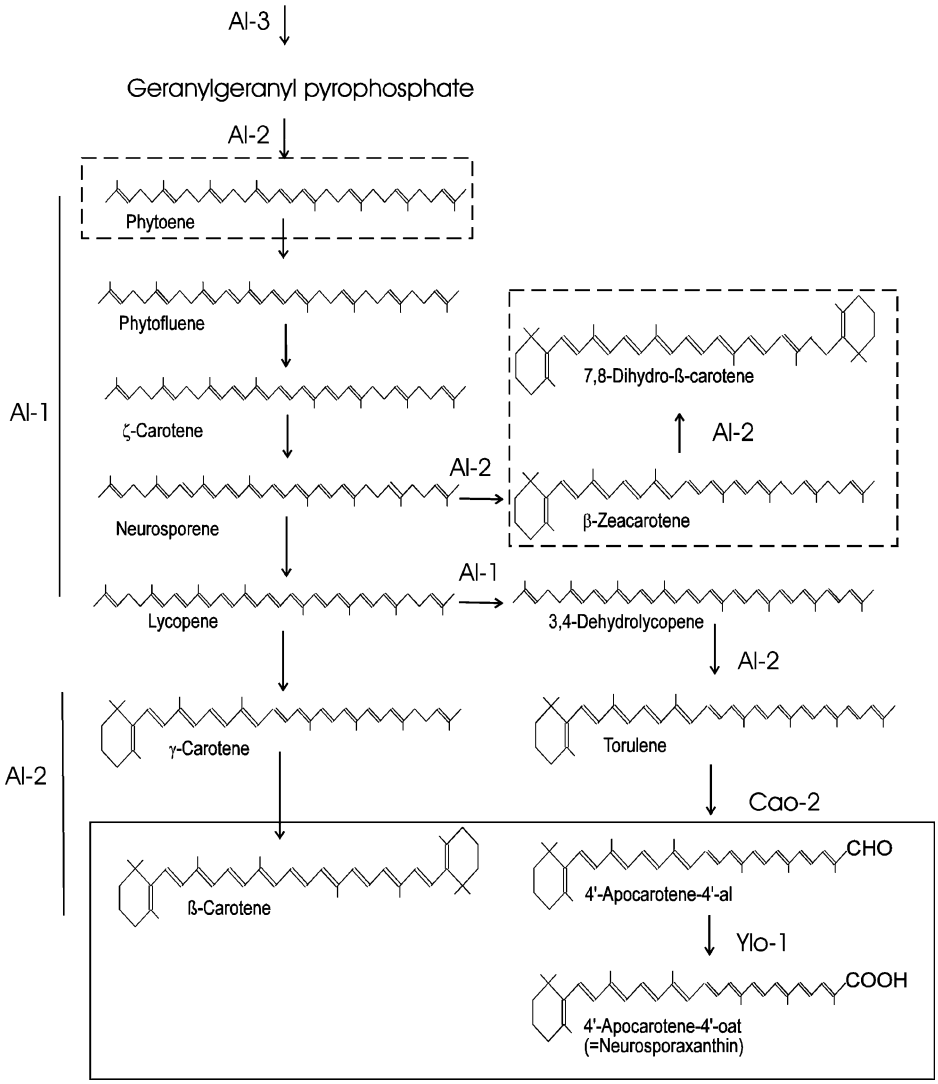

Fig. 2 Carotenogenic pathway of *Podospora anserina* and transformants. *Boxed* carotenoids are the products in WT, carotenoids in *dotted box* accumulate in transformants with over-expressed *PaAl-2* gene. Gene products catalyzing the individual reactions are indicated, nomenclature was applied according to the homologous *Neurospora crassa* genes

(peak at 12 min of dotted trace recorded at 285 nm, maxima at 275, 285 and 297 nm) and neurosporene (at 52 min, maxima at 414, 440 and 467 nm) as well as the cyclic β-zeacarotene (at 25 min, maxima at 405, 428 and 455 nm) and 7,8-dihydro-β-carotene (at 31 min, maxima at 405, 428 and 455 nm). These structures are marked in Fig. 2. The double mutant PaAl-1_Ex/PaAl-2_Ex synthesized the same types of carotenoids as PaAl-1_Ex alone except for some additional β-carotene and phytoene which is not shown here. The other double mutant PaAl-2_Ex/PaAl-3_Ex exhibited the same qualitative carotenoid composition as PaAl-2_Ex.

The carotenoids of the transformants were quantitated in Table 1. The concentrations of the carotenoids are quite low when comparing to *N. crassa* in which the carotenoids levels are about 50-fold higher (Sandmann et al. 2008). Over-expression of *PaAl-1* alone had no effect on total carotenoids but β-carotene dropped below detection and small amounts of torulene were found. A similar effect was observed upon over-expression of *PaAl-3*. But here β-carotene was little affected and small amounts of γ-carotene instead of torulene were present.

The strongest influence on the carotenoid composition was found with PaAl-2_Ex. Total carotenoids increased more than 600-fold indicating that the phytoene synthase reaction was enhanced. The dominating carotenoid was phytoene but the concentration of neurosporaxanthin was hardly affected. Intermediates of the pathway like neurosporene, lycopene, γ-carotene and torulene accumulated. In addition, β-zeacarotene and 7,8,-dihydro-β-carotene, the mono and dicyclic products of neurosporene, were synthesized. The combination of *PaAl-2* with *PaAl-1* yielded more than 70-fold higher neurosporaxanthin concentrations when compared with the wild type at the expense of phytoene, which was lower than in PaAl-2_Ex. In PaAl-1_Ex/PaAl-2_Ex, the highest torulene concentrations were found. The double mutant PaAl-2_Ex/PaAl-3_Ex but the yield of all accumulating carotenoids were substantially lower than in the culture with over-expressing *PaAl-2* alone.

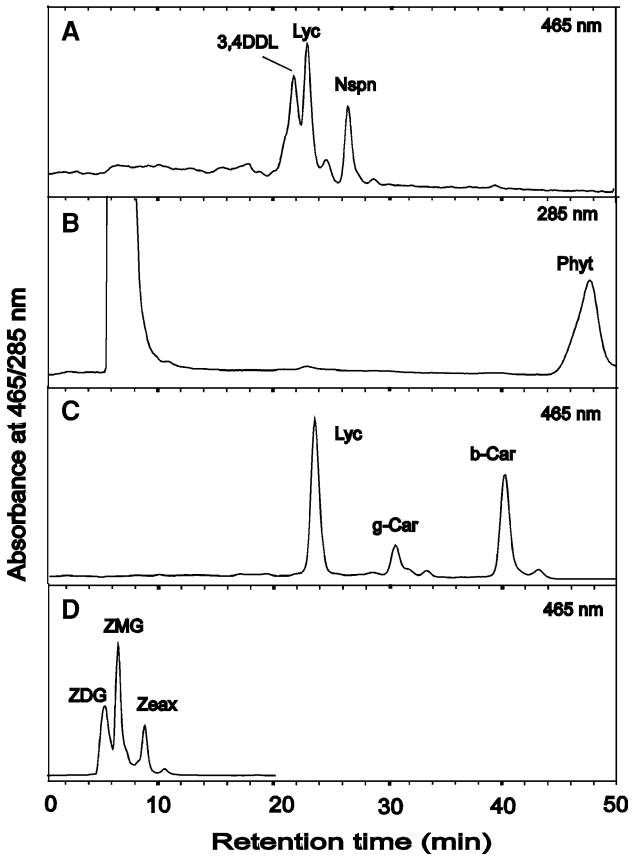

Fig. 3 Functional assignment by complementation in *E. coli* of the carotenogenic genes from *Podospora anserina*. HPLC: **a** *E. coli*/pMONal-1/pACCRT-EB, **b** *E. coli*/pMONal-2/pACCRT-E, **c** *E. coli*/pMONal-2/pACCRT-EBI and **d** *E. coli*/pUC8al-3/pACCAR25ΔcrtE. Separation was on a Hypersil HyPurity Elite C18 3μ column with acetonitrile/methanol/2-propanol (85:10:5, by volume). *Nspn* neurosporene, *Lyc* lycopene, *Phyt* phytoene, *g-Car* γ-carotene, *b-Car* β-carotene, *Zeax* zeaxanthin, *ZMG* zeaxanthin monoglucoside, *ZDG* zeaxanthin diglucoside

The remarkable concentrations of neurosporaxanthin and other carotenoids in the PaAl-1_Ex/PaAl-2_Ex double mutant lead to a dramatic alteration of mycelial pigmentation on solid medium, cornmeal agar, and in liquid culture. Also the envelope of the perithecia (i.e. fruiting bodies) is characterized by a red-orange colouration.

Lifespan extension in transgenic *P. anserina* strains

Since the *PaAl-2* over-expressing mutants PaAl-2_Ex, PaAl-1_Ex/PaAl-2_Ex and PaAl-2_Ex/PaAl-3_Ex display the greatest impact on carotenoid composition and concentrations, we anlalyzed the effect of these molecular modifications on the life span of these strains. Survival curves were determined in race tubes filled with 50 ml of growth medium, PASM + 1% glucose (Fig. 6).

Progeny of primary transformant PaAl-2_Ex T7 ($n = 42$) are characterized by an increased of mean life span by 31% compared to the wild type control ($n = 63$, $p < 4.29 \times 10^{-7}$, Wilcoxon test). Meiotic offspring of the double mutants PaAl-1_Ex/PaAl-2_Ex and PaAl-2_Ex/PaAl-3_Ex ($n = 42$ each) is also long-lived, displaying a 16% (PaAl-1_Ex/PaAl-2_Ex) and 21% (PaAl-2_Ex/PaAl-3_Ex) extension of the mean life span, respectively. Remarkably, the mean growth rate of the investigated mutants is significantly increased compared to the wild type (PaAl-2_Ex: +26.4%, $n = 20$, $p \leq 7.4 \times 10^{-9}$; PaAl-1/PaAl-2_Ex: +6.1%, $n = 20$, $p \leq 0.0003$; PaAl-2/PaAl-3_Ex +23.7%, $n = 16$, $p \leq 1.12 \times 10^{-7}$).

Discussion

Carotenoid biosynthesis in *P. anserina*

This study demonstrated that *P. anserina* is a carotenogenic fungus (Fig. 1). It synthesizes neurosporaxanthin and β-carotene as two end products of the pathway. However, the yields are quite low (Table 1) when compared with other fungi like *N. crassa* (Sandmann et al. 2008). The over-expressing mutants revealed the bottle-necks of the pathway in *P. anserina*. The major limitation is in phytoene synthesis which is overcome when *PaAl-2* is over-expressed. In the PaAl-2_Ex transformant, mainly phytoene accumulated indicating that also the desaturation reactions are limiting in *P. anserina*. This is also the case in *N. crassa* where phytoene is the major carotenoid and most intermediates to β-carotene and torulene dominate over the end product neurosporaxanthin (Sandmann et al. 2008). In the double mutant PaAl-1_Ex/PaAl-2_Ex of *P. anserina* with an additional phytoene desaturase, phytoene is efficiently metabolized through the entire pathway leading to higher concentrations of the end products β-carotene and neurosporaxanthin (Table 1). Accumulation of torulene in PaAl-1_Ex/PaAl-2_Ex indicates that the cleavage reaction (Fig. 2) catalyzed by CaO-2 (Saelices et al. 2007) is the next enzyme exhibiting a moderate limitation in a pathway with over-expressed *PaAl-2* and *PaAl-1*. Obviously, there is no limitation in geranylgeranyl pyrophosphate provision even in PaAl-2_Ex, since PaAl-2_Ex/PaAl-3_Ex shows no increase of total carotenoids over PaAl-2_Ex.

The first three genes for the carotenogenic pathway have been cloned from *P. anserina* and functionally assigned (Fig. 3). They resemble all genes necessary for the biosynthesis branch to β-carotene (Fig. 2). Their similarities to the corresponding genes from *N. crassa* are high. In addition, sequences homologous to the *cao-2* (Saelices et al. 2007) and *ylo-1* (Estrada et al. 2008) genes for the final steps to neurosporene are present in the sequenced genome from *P. anserina* (Espagne et al. 2008). Their cloning and analyses is in progress.

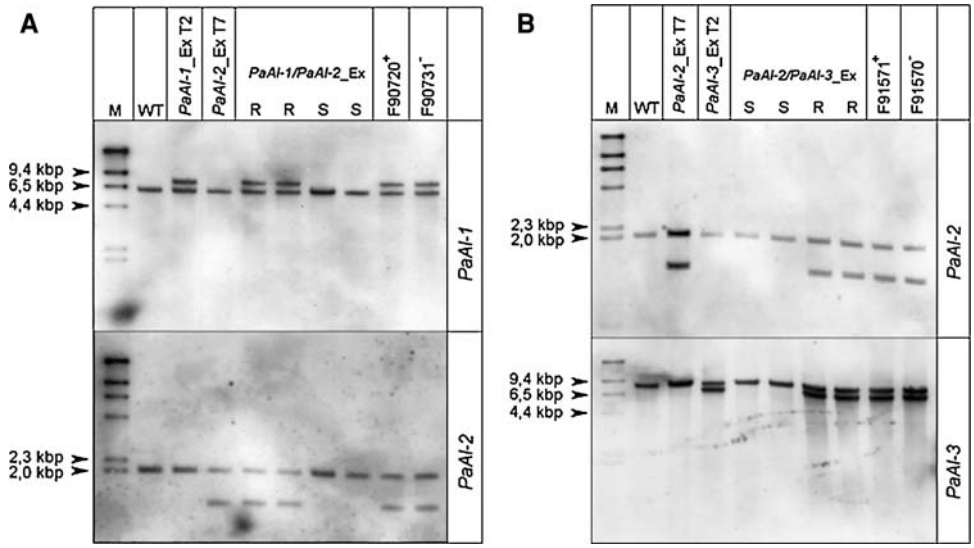

Fig. 4 Validation of transgenic *Podospora anserina* strains by Southern blot analysis. **a** Transformants PaAl-1_Ex T2, PaAl-2_Ex T7 and double mutant PaAl-1_Ex/PaAl-2_Ex hybridized with *PaAl-1*- and *PaAl-2*-specific probes. F90720^{+} and F90731^{-} are double mutants derived from germinated monokaryotic spores. **b** Transformants PaAl-2_Ex T7, PaAl-3_Ex T2 and double mutant PaAl-2_Ex/PaAl-3_Ex hybridized with *PaAl-2*- and *PaAl-3*-specific probes. F91571^{+} and F91570^{-} are double mutants germinated from monokaryotic spores. Double mutants were created by crossing the single mutants. The DNA preparations used in these studies were digested with *Hin*dIII. *M* DIG-labelled λ *Hin*dIII marker, *WT* genomic wild type DNA, + *and* − mating type of the isolate, *R or S* indicates hygromycin B resistance or sensitivity

Biotechnological potential of *P. anserina* for the synthesis of carotenoids

Although the wild type of *P. anserina* exhibits low carotenogenesis, we demonstrated that this fungus can be genetically engineered towards much higher carotenoid production (Table 1). Transformants with over-expressing *PaAl-2* gene are equal or even superior to the fungi *Candida utilis* (Miura et al. 1998), *Pfaffia rhodozyma* (Verdoes et al. 2003) and *Saccharomyces cerevisiae* (Verwaal et al. 2007) all engineered for optimum carotenogenesis. Due to the limitations of its desaturation pathway which is mainly directed to 3,4-dehydrolycopene and resulting mono-cyclic products (Fig. 3), *P. anserina* can easily be modified to synthesize bi-cyclic carotenoids by using a heterologous phytoene desaturase gene-like *crtI* encoding a four-step desaturase. This will direct the pathway away from neurosporaxanthin synthesis to β-carotene (Fig. 2). Such a transformant should have the potential to synthesis up to 2 mg/g dw of β-carotene or related carotenoids with high demand like astaxanthin or zeaxanthin. This yield may even be considerably increased by engineering the supply of precursors for carotenogenesis as exemplified in *C. utilis* (Miura et al. 1998) and *S. cerevisiae* (Verwaal et al. 2007). In the latter case, over-expression of a 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene in a transformant synthesizing similar amounts of carotenoids like PaAl-1_Ex/PaAl-2_Ex resulted in an additional improvement of the carotenoid production by a factor of 3.

Carotenoids affect the ageing process in *P. anserina*

All lines over-expressing *PaAl-2* exhibited a significantly longer mean lifespan than the wild type (Fig. 6). Ageing in *P. anserina* is connected to the generation of free radicals leading to oxidative cell damage by ROS (Harman 1992) whereas most carotenoids are able to quench singlet oxygen and inactivate oxygen radicals (Edge et al. 1997). Therefore, one can hypothesize that the antioxidative properties of the carotenoids accumulating in the transformants play the decisive role in protection. Except for the mutual high level of phytoene, all three mutants possess different carotenoids in different concentrations (Fig. 5, Table 1). Common to them is also an increased concentration of β-carotene, which is at least 3.8-fold higher than in the wild type. Due to the rather short conjugated double bond system, phytoene cannot function as an effective antioxidant. However, transgenic mice which produced phytoene acquired resistance against oxidative stress and oncogenic transformation (Satomi et al. 2004). Therefore, phytoene in addition to β-carotene can be regarded as another candidate, which may be involved in the prolongation of the life span of *P. anserina* transformants. In another intensively studied ageing model, *Drosophila melanogaster*, it was recently reported that the supplementation of SOD1-deficient flies with the carotenoid derivative retinol (vitamin A) leads to a concentration dependent extension of life span (Bahadorani et al. 2008).

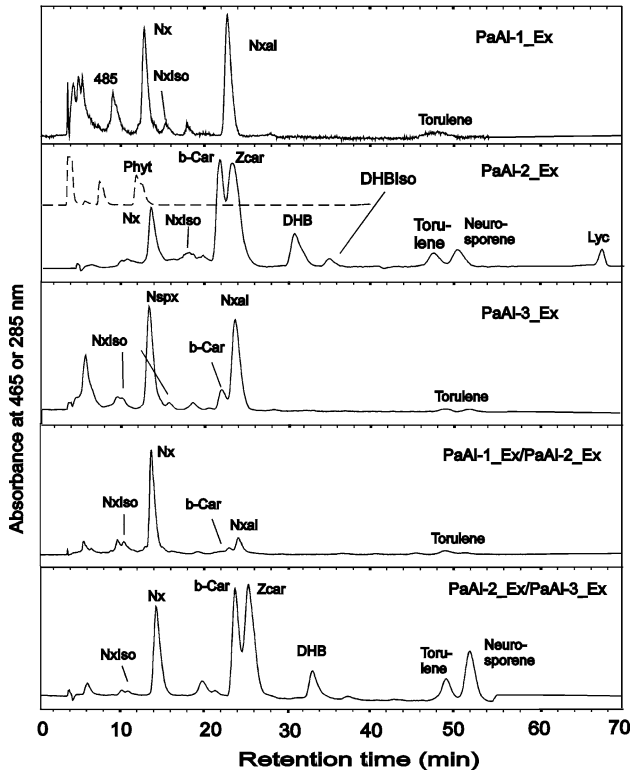

Fig. 5 HPLC separation of carotenoids in *Podospora anserina* transformants with engineered carotenogenic pathway. PaAl-1_Ex with over-expressed *PaAl-1* gene, PaAl-2_Ex with over-expressed *PaAl-2* gene, PaAl-3_Ex with over-expressed *PaAl-3* gene, and the double mutants PaAl-1_Ex/PaAl-2_Ex and PaAl-2_Ex/PaAl-3_Ex. Separation was on a C30 column eluted with methanol/methyl-*tert*-butyl ether/water. *Nx* neurosporaxanthin, *Nxal* 4′-apo-γ-carotene-4′-al, *b-Car* β-carotene, *Zcar* β-zeacarotene, *DHB* 7,8-dihydro-β-carotene, 485 corresponds to an acidic carotenoid with maxima at 455, 485 and 515 nm. *Iso* indicates cis isomers

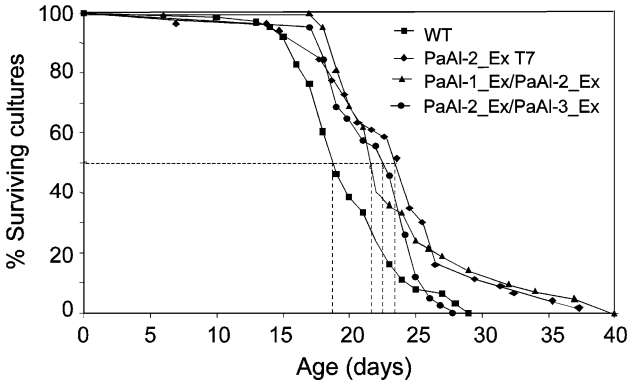

Fig. 6 Determination of life spans of *Podospora anserina* transformants PaAl-2_Ex T7, PaAl-1_Ex/PaAl-2_Ex and PaAl-2_Ex/PaAl-3_Ex over-express the *PaAl-2* gene alone or in combination with *PaAl-1* and *PaAl-3*. Numbers of cultures were 63 for wild type and 42 for all transformants

Whether either the function of phytoene or the metabolic conversion of carotenoids is relevant for *P. anserina* ageing remains open. The most likely explanation for the protection from ageing in the *PaAl-2* over-expressing transformants is by a synergistic antioxidative effect of different carotenoid combinations in which β-carotene may play a prominent role. Currently, it is unknown whether energy metabolism or mitochondrial function, which are known to crucially are involved in ageing in *P. anserina* (for a review see Osiewacz 2002), are influenced in the transgenic *P. anserina* strains investigated in this study. However, the significantly increased growth rates of the mutants suggest a beneficial effect on their overall metabolic activity.

The demonstrated protection against ageing in the *PaAl-2* over-expressing transformants can be regarded as an initial

Table 1 Carotenoid content (μg/g dry weight) of *Podospora anserina* wild type and transformants

Car.	WT	PaAl-1 Ex T2	PaAl-2 Ex T7	PaAl-3 Ex T2	PaAl-1_Ex/PaAl-2_Ex	PaAl-2_Ex/PaAl-3_Ex
Nx	18.3 ± 1.5	14.1 ± 2.4	22.9 ± 3.6	19.9 ± 2.2	1,398.0 ± 567.6	11.6 ± 5.5
NxAl	13.6 ± 0.7	19.7 ± 8.1	21.0 ± 6.1	15.5 ± 2.5	382.9 ± 140.5	tr
Lyc	nd	nd	13.4 ± 2.1	nd	nd	nd
Nn	nd	nd	8.5 ± 1.7	nd	nd	6.5 ± 2.0
Tor	tr	0.9 ± 0.7	11.0 ± 4.4	nd	251.4 ± 220.1	2.8 ± 1.3
g-Car	nd	nd	tr	1.2 ± 0.1	nd	nd
b-Car	2.8 ± 0.2	nd	37.5 ± 6.9	2.2 ± 0.1	55.0 ± 15.4	10.7 ± 3.2
Zcar	nd	nd	11.1 ± 2.8	nd	nd	14.9 ± 4.4
DHB	nd	nd	23.4 ± 2.9	nd	nd	3.6 ± 1.1
Phyt	nd	nd	2,735.6 ± 698.8	nd	94.7 ± 28.1	401.5 ± 257.2
Total	34.7	34.7	2,884.4	38.8	2,182.0	451.6

Nx neurosporaxanthin, *NxAl* 4′-apo-γ-carotene-4′-al, *Lyc* lycopene, *Nn* neurosporene, *Tor* torulene, *g-Car* γ-carotene, *b-Car* β-carotene, *Zcar* β-zeacarotene, *DHB* 7,8-dihydro-β-carotene, *Phyt* phytoene, *nd* not detectable

Values are means of 5–8 determinations ± SD from different cultures

In transformant PaAl-1_Ex T2 an acidic carotenoid (4.6 ± 2.0 μg/g dw) was found with absorbance maxima at 455, 485 and 515 nm, which may correspond to 4′-apo-lycopene-4′-oate

result. It merits future studies focused on a detailed analysis of cellular processes related to ageing, the antioxidant status and the sites of protection in the generated transformants. Furthermore, it is possible to engineer for the synthesis of specific carotenoids, which is well advanced in bacteria (Sandmann 2002) and reveal a relationship between the life span and the presence of special carotenoid structures.

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