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Accumulation of ζ-carotene in *Chlamydomonas reinhardtii* under control of the *ac5* nuclear gene

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Abstract The accumulation of different precursors of carotenoid biosynthesis in carotenoid-deficient mutants of *Chlamydomonas reinhardtii* was studied by HPLC-analysis. ζ-Carotene accumulated in several *ac5* mutants, this character cosegregated with mutations in the *ac5* gene. Two groups of *ac5* mutants differing in ζ-carotene accumulation were distinguished. One (*ac5*–1) accumulated ζ-carotene in the dark but not in the light. The other (*ac5*–2) accumulated ζ-carotene under both dark and light conditions. *ac5*–2 strains accumulated more ζ-carotene in the dark than *ac5*-1 strains. Genetic data suggested that the mutations *ac5*–1 and *ac5*–2 were allelic. Pleiotropic effects of mutations in the *ac5* gene included decreased levels of chlorophyll a and b and acetate requirement. The results are consistent with the presence of a defective ζ-carotene desaturase in *ac5* mutants.

Key words Chloroplast biogenesis · Carotenoid biosynthesis · *Chlamydomonas reinhardtii* · ζ-Carotene

Introduction

The unicellular green alga *Chlamydomonas reinhardtii* is very often used as a model for chloroplast genetics and biogenesis (Rochaix 1995). As in many other photosynthesizing organisms, genetic studies began with the isolation of pigment mutants (Ebersold 1956). Originally, the *C. reinhardtii* strain carrying a mutation in the gene *ac5* was isolated as a non-autotrophic, acetate-requiring, pigment-deficient strain *na*. It was shown to be a single-gene mutation, inherited in a Mendelian fashion (Ebersold 1956). The *ac5* mutation was localised in linkage group VII of the nuclear genome (Levine and Goodenough 1970). Later, strains of this genotype could be grown in other laboratories either phototrophically in the light on a minimal medium or mixotrophically in the light on a minimal medium supplemented with sodium acetate. Under both growth conditions, strains carrying an *ac5* mutation produced cells with reduced chlorophyll content, particularly chlorophyll b, compared to wild-type cells. The photosynthetic capacity of *ac5* cells was comparable under both growth conditions (Goodenough and Staehelin 1971).

Until recently, no information was available on the biochemical reactions under the control of the *ac5* gene. In this article, the accumulation of ζ-carotene in several *ac5* mutant strains is described and data on (a) the light dependency of ζ-carotene accumulation, (b) the number of alleles affecting ζ-carotene accumulation and (c) the pleiotropic effects of these mutations are reported.

Materials and methods

C. reinhardtii strains and growth methods

C. reinhardtii ac5 strains CC-1677 and GB-1 (Chlamydomonas Genetics Center, Duke University, USA), C41 and A110 (Ladygin 1991), as well as segregants obtained during our work were used. Strains were cultivated either in liquid medium (Sager and Granick 1954) supplemented with Na-acetate (2 g/l) or on solid media containing 1.5% agar. The acetate requirement of strains was tested on different solid media, such as TAP, Sager and Granick, or Levine and Ebersold in the presence or absence of acetate (for composition of media see Harris 1989). Induction of gametes, crossings, maturation of zygotes and dissection of tetrads were carried out according to Levine and Ebersold (1960). Cell numbers from liquid cultures were counted in a haemocytometer and chlorophyll was determined as described by Vernon (1960).

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Pigment analysis

For pigment analysis, cells from liquid cultures were harvested at the end of the exponential growth phase at about $1–4 \times 10^6$ cells/ml. A volume equivalent to 44 mg chlorophyll was centrifuged and the cells were extracted with 80% acetone. The pigments were transferred into diethyl ether. After evaporation of the solvent under a stream of nitrogen, the pigments were dissolved again in about 20 ml 100% acetone and analysed by HPLC under conditions for optimal separation of carotenoid hydrocarbons (Nucleosil5-C18; 250/4 mm; Macherey-Nagel, Oensingen, Switzerland), with acetonitrile:methanol:isopropanol 85:10:5 (by vol) as solvent, flow rate 1.5 ml/min for 6 min, 0,5 ml/min for 30 min. Chlorophylls and carotenes were detected at 425 nm corresponding to an absorption maximum of ζ-carotene. Peak fractions were collected and identified spectrophotometrically as chlorophyll a, chlorophyll b, ζ-carotene and β-carotene (Fig. 1). The amount of ζ-carotene was calculated using an extinction coefficient in this solvent at 425 nm of E (1 mg/ml) = 227.

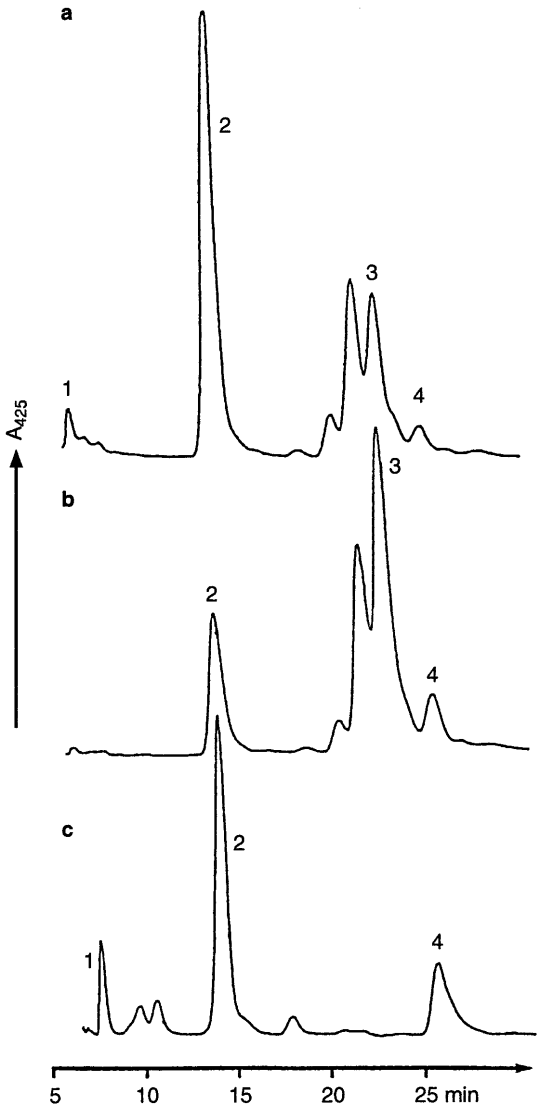

Fig. 1 HPLC separation of pigment extracts from dark-grown GB-1 mutant (**a**), CC-1677 (**b**), and wild-type strain (**c**) (*1* chlorophyll b, *2* chlorophyll a, *3* ζ-carotene, *4* β-carotene). Detection at 425 nm

For rapid screening of numerous strains for ζ-carotene accumulation, spectra of the acetone extracts of the algal cells were recorded (Specord UV-VIS). The presence of three peaks in the region between 380 and 430 nm pointed to an accumulation of ζ-carotene in the strain tested.

Results and discussion

Detection of ζ-carotene in *ac5* mutants

The pigment composition of *ac5* mutants of *C. reinhardtii* was studied first using the strains GB-1 and CC-1677. These mutants formed small pale-green colonies in the light and yellow-white colonies in the dark. Pigment analysis by HPLC of dark-grown cultures revealed an accumulation of ζ-carotene, i.e. of a pale-yellow biosynthetic precursor of carotenoids (Fig. 1). As the *ac5* mutation was originally detected by its acetate requirement, the strains were tested on different growth media. All *ac5* mutants used in this study could indeed grow phototrophically, but much slower than wild-type strains. Obviously, these *ac5* strains had no absolute requirement for acetate, but growth was highly stimulated by it. Therefore, the phenotype of the strains GB-1 and CC-1677 *ac5* could be defined by (1) acetate requirement, (2) ζ-carotene accumulation and (3) reduced greening of the colonies.

Quantitative pigment analysis confirmed the low content of coloured carotenoids, e.g. β-carotene, as well as the increased accumulation of the precursor ζ-carotene in dark-grown strains GB-1 and CC-1677, compared to wild-type strains (Figs. 2, 3). Furthermore,

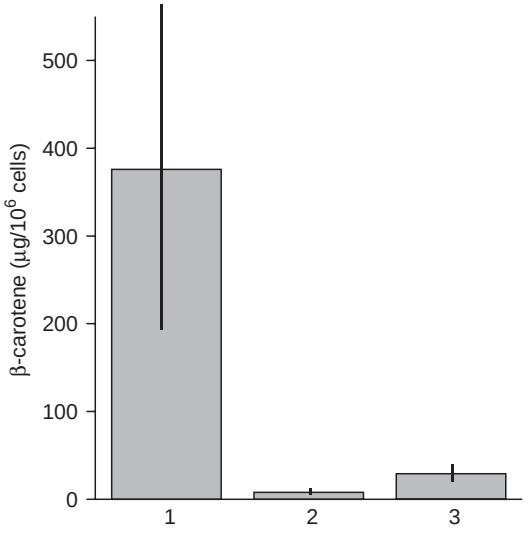

Fig. 2 Comparison of β-carotene content in dark-grown cultures of wild-type and *ac5* mutant strains. Mean values of β-carotene content in phenotypically wild-type strains (*1*), *ac5*–1-type mutants, i.e. CC-1677, GB1 and mutant segregants from cross 1656 (*2*), and *ac5*–2-type strains, i.e. C41 and mutant segregants from cross no. 1661 (*3*) (*bar* 95% confidence interval from *t*-test)

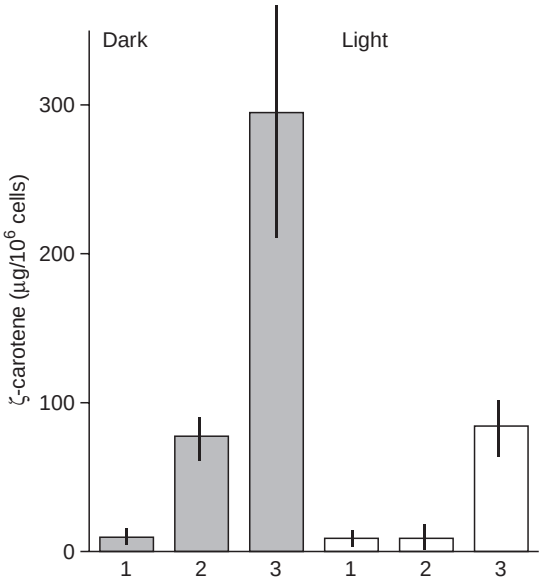

Fig. 3 Comparison of ζ-carotene content of wild-type and *ac5* mutant strains grown in dark or light. Mean values of ζ-carotene content in phenotypically wild-type strains (*1*), *ac5*–1-type mutants, i.e. CC-1677, GB1 and mutant segregants from cross 1656 (*2*), and in *ac5*–2-type strains, i.e. C41 and mutant segregants from cross no. 1661 (*3*) (*bar* 95% confidence interval from *t*-test)

a similarly reduced formation of β-carotene was also found in strain C41 (see below) and in segregants from crosses of such *ac5* strains with wild-type strains (Fig. 2). Absorption spectra taken by a photodiode array detector during an HPLC run suggested that in mutant segregants, three stereoisomers of ζ-carotene were present and could be separated (not shown).

Cosegregation of ζ-carotene accumulation with *ac5* phenotypes

To verify whether the *ac5* gene is responsible for the accumulation of ζ-carotene, the cosegregation of the *ac5* phenotype with ζ-carotene accumulation in the progeny of crosses of wild-type with *ac5* mutants was studied (Table 1). Analysis of complete tetrads as well as random samples of progeny from cross no. 1656, i.e. CC-1677(*ac5*) × CC-124(wt), demonstrated that the *ac5* phenotype was inherited in a Mendelian fashion. The progeny of this cross were used to study the pigment composition of five complete tetrads. Each tetrad consisted of two clones showing wild-type phenotype and two others with *ac5* phenotype, accumulating ζ-carotene (Fig. 3). Random samples from crosses giving incomplete tetrads were also analysed. Crosses between progeny clones showed that ζ-carotene accumulation cosegregated with the mutation in the *ac5* gene, again in a Mendelian fashion (Table 1, cross no.1904). It is concluded that in *C. reinhardtii*, mutations in the gene *ac5* (linkage group VII) cause accumulation of ζ-carotene due to a defect in ζ-carotene desaturase. *ac5* mutants might therefore be used for isolating the gene for this enzyme from this organism.

The character of the *ac5* mutation was further studied by crossing a double-mutant strain carrying both the *arg7-8* and an *ac5* mutation with a strain carrying the *arg7-3* mutation (Table 2, strains 1904-8 and CF-30). Among the progeny of this cross, three vegetative diploids were selected. All of them grew on a medium without arginine as a result of intra-allelic complementation of *arg7* mutant alleles. They were of mating type "minus" and had a larger cell volume than wild-type haploid cells (Table 2). No ζ-carotene accumulation was observed in these diploid strains, indicating that *ac5* is a recessive mutation.

Phenotypic characterisation of additional ζ-carotene-accumulating mutants

Pleiotropic effects of mutations in the *ac5* gene included decreased levels of chlorophyll a and b (Fig. 4), possibly as a result of defective assembly of light-harvesting complexes in the absence of carotenoids (Herrin et al. 1992). This chlorophyll deficiency, which always cosegregated with acetate requirement and with ζ-carotene accumulation, offered a simple selection method for the identification of mutant strains. By observation of chlorophyll fluorescence through a red broad-band glass filter from colonies illuminated by blue-green light, *ac5* mutant colonies showed much weaker fluorescence than wild-type colonies (Chunaev et al. 1991).

On the basis of these characteristics, the Peterhof Genetic Collection of Microorganisms (Samsonova et

Table 1 Genetic analysis of *ac5* mutants [(*ac5*) mutation in *ac5* gene, (*?*) unknown genotype, *Ac5* phenotype of *ac5* mutation, (*wt*) wild-type genotype, *Wt* phenotype of wild-type]

No. of cross	Parent mt+	Parent mt−	Full tetrads	Random samples
1656	CC-1677(ac5)	CC-124	5 tetrads 2*ac5*:2 wt	12 Ac5:13 Wt
1661	C41(?)	494(wt)	2 tetrads 2*ac*:2 wt	–
1901	1661-16a(?)	1656-4a(wt)	1 tetrad 2*ac*:2 wt	21 Ac5:24 Wt
1904	1656-1d(ac5)	1665 1b(wt)	–	195 Ac5:163 Wt
1905	1656-1d(ac5)	1661-16b(?)	–	607 Ac5
1906	1656-1d(ac5)	1656-5b(ac5)	–	9468 Ac5

Table 2 Description of diploid strains carrying *ac5* mutations, parental strains and wild-type strains. From the parental strains 1904-8 and CF-30, the diploid strains D1, 2, 3 were obtained. The diploids are not arginine dependent due to intra-allelic complementation. The cell volume was calculated using data from micrographs and the formula: $V = 1/6 \cdot \pi \cdot d_{max} \cdot d_{min}$ (μm^3)

Name of strain	Ploidy	Genotype	Growth without arginine	Mating type	Cell volume (mm^3)	Accumulation of ζ-carotene
D1-1918	2n	arg7-8ac5/arg7-3	+	−	271.8 ± 57.6	−
D2-1918	2n	arg7-8ac5/arg7-3	+	−	530.2 ± 33.1	−
D3-1918	2n	arg7-8ac5/arg7-3	+	−	390.4 ± 14.8	−
1904-8	n	arg7-8ac5	−	+	180.3 ± 13.2	+
CF-30	n	arg7-3	−	−	154.3 ± 9.7	−

al. 1994) was screened for other *Chlamydomonas* strains which accumulated ζ-carotene. The two strains C41 and A110 were found to behave as *ac5* strains and showed similar characteristics. Strain C41 (genotype originally unknown; Ladygin 1991) was studied in more detail. It formed pale-green colonies and accumulated ζ-carotene as described for *ac-5* strains. In the progeny of crosses of C41 with wild-type, three complete tetrads were isolated showing Mendelian phenotype inheritance (Table 1, crosses no. 1661 and 1901). The pigments of the progeny of two of these tetrads were analysed by HPLC. The parental strain C41 and the segregants carrying the mutation accumulated more ζ-carotene than the original *ac5* mutants GB1 or CC-1677, both in dark and light (Fig. 3). Furthermore, in cells grown either in light or dark, the chlorophyll a and chlorophyll b contents were reduced compared to the wild-type strains (Fig. 4), but comparable with that in *ac5* strains.

The phototrophic growth rate of C41 was similar to that of CC-1677 and GB-1, but among the progeny of cross no. 1661, some segregants were completely unable to grow in the light without sodium acetate. The acetate requirement, which was originally used as an *ac5* character, is not directly linked to defects in photosynthesis, as the photosynthetic capacity was found to be comparable under both phototrophic and mixotrophic growth conditions (Goodenough and Staehelin 1971). The occurrence of some segregants among the progeny of crosses between C41 and wild-type showing strong acetate requirement in the light might suggest the presence of a gene suppresser in C41 which partially overcame the acetate requirement without affecting other characteristics of the *ac5* mutation. Based on the similarity of its phenotype and ζ-carotene accumulation, strain A110 which was derived from 137c– (wt, mt–) by X-ray irradiation by Ladygin et al. (1991), is also considered to be an *ac5* gene mutant.

The description of the ultrastructure of the chloroplasts in the C41 mutant was very similar to that found in *ac5* mutants with respect to the predominance of unstacked thylakoids (Goodenough and Staehelin 1971; Ladygin et al. 1979). Defects in thylakoid stacking might be related to alterations in or incorrect assembly of light-harvesting complexes due to altered carotenoid composition. The reduced chlorophyll content in ζ-carotene-accumulating cells might be similarly explained. However, other mechanisms for coregulation of carotenoid and chlorophyll biosynthesis are not excluded (Paulsen 1997). Strains carrying mutations in the *ac5* gene are not light sensitive and grow phototrophically, although more slowly than the wild-type. Under mixotrophic conditions, *ac5* mutants contain coloured carotenoids, but on solid media, the colonies

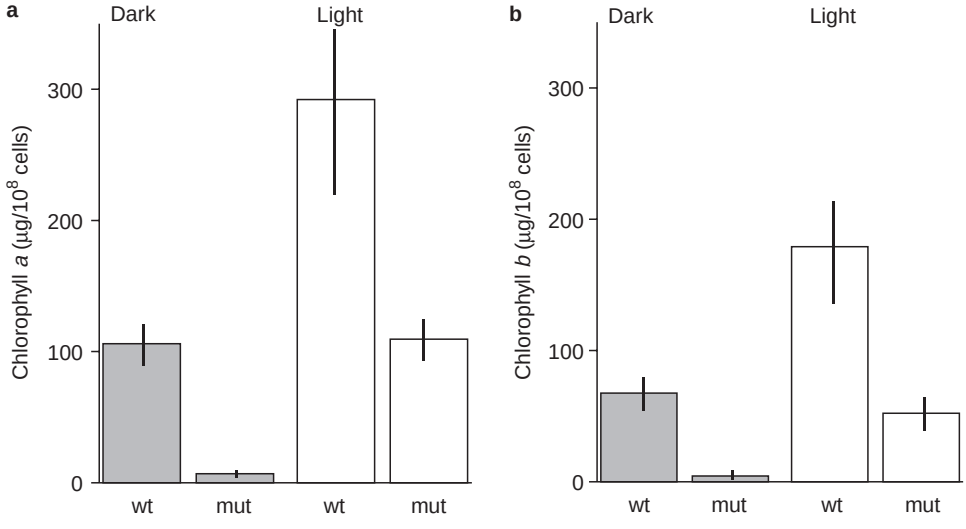

Fig. 4 Comparison of chlorophyll content in wild-type strains and *ac5* mutants. Mean values of all determinations of chlorophyll a (**a**) or chlorophyll b (**b**) content in phenotypically wild-type strains (*wt*) and in all ζ-carotene-accumulating strains (*mut*) (*bar* 95% confidence interval from *t*-test)

are small and pale green as a result of the reduced chlorophyll content. In higher plants, light-induced formation of carotenoids has been ascribed to phytochrome-mediated expression of phytoene synthase. A transcriptional, probably end-product regulation of phytoene desaturase has also been observed in higher plants (Corona et al. 1996; von Lintig et al. 1997). The behaviour of the *ac-5* strains of *Chlamydomonas* which lack phytochrome might suggest a light-dependent, but not phytochrome-dependent regulation of ζ-carotene desaturase.

Allelic relationship between *ac5* mutants

To examine for allelic relationships between the C41 mutation and the *ac5* mutation, a recombination test was used (Table 1, cross no. 1905). Lethality in the progeny of crosses, however, was high and we were unable to obtain complete tetrads. On the basis of results from random samples of tetrads it could be concluded that both the mutation in the C41 strain and that in the *ac5* strains were confined to one gene. In the positive-control cross no. 1906 (Table 1), wild-type segregants were also absent from the progeny. We designated as *ac5*–1, the group of *ac5* strains isolated by Ebersold, and *ac5*–2, to the C41-group mutants. The first group (*ac5*–1) accumulated ζ-carotene in the dark, but not in the light. The second group (*ac5*–2), accumulated ζ-carotene in both dark and light (Fig. 3). The β-carotene content was also reduced to a different degree in the two groups (Fig. 2). The *ac5*–1 group accumulated less β-carotene and ζ-carotene in the dark than the *ac5*–2 group. The low content of β-carotene and chlorophylls (Figs. 2, 4) probably reflects a more severe impairment of photosynthetic membranes in *ac5*–1 than in *ac5*–2 mutants. The lower accumulation of ζ-carotene in *ac5*–1 strains might point to the photosynthetic membrane as the site of ζ-carotene accumulation.

In recent years, plant genes encoding enzymes of carotenoid biosynthesis have been cloned using various approaches (Scolnik and Bartley 1996). Genes that participate in control of the ζ-carotene-desaturating enzyme in plants (*Arabidopsis thaliana, Capsicum annuum* and *Oryza sativa*) and cyanobacteria, have been identified and cloned (Sasaki et al. 1994; Albrecht et al. 1995; Scolnik and Bartley 1995; Breitenbach et al. 1998). In *Chlamydomonas*, the genetic control of carotenoid synthesis has not been studied in detail, although a number of light-sensitive mutants defective in carotenoid biosynthesis exist. Additionally, several norflurazon-resistant mutants accumulating phytoene were recently found (Zvinchouk AR, unpublished) and the gene *lor1* was shown to block the synthesis of loroxanthin, a specific algal xanthophyll, by preventing α-cyclisation. (Michel et al. 1983; Chunaev et al. 1991). With the *ac5* mutants described here, an additional gene involved in the control of carotenoid biosynthesis becomes accessible. The *ac-5* mutation, when incorporated into heterozygotic diploids together with the wild-type allele, may enable isolation of the gene for ζ-carotene desaturase from *C. reinhardtii*.

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