Complete separation of the β,ε- and β,β-carotenoid biosynthetic pathways by a unique mutation of the lycopene cyclase in the green alga, *Scenedesmus obliquus*

Norman I. Bishop[a], Thomas Urbig[b], Horst Senger[b,*]

[a]*Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, USA*
[b]*Fachbereich Biologie/Botanik, Philipps-Universität Marburg, Lahnberge, D-35039 Marburg, Germany*

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Abstract The mutant, C-2A′-34, lacks the β, ε-carotenoids, α-carotene, lutein and loroxanthin. When grown under heterotrophic or mixotropic conditions this strain develops significantly higher levels of β-carotene and violaxanthin than does the original developmental mutant of *Scenedesmus*, C-2A′. The decrease in chlorophyll *a* and chlorophyll *b* observed in C-2A′-34 is accompanied by the near absence of the LHC. The light intensity dependence of greening of this strain is comparable to that of C-2A′; the loss of the β,ε-carotenoids and modification of the pool of β,β-carotenoids neither prevent the proximal pigment–protein complexes of photosystems I and II from developing nor cause any short term photosensitivity. The increase in the β,β-carotenoids in C-2A′-34 apparently compensates for the loss of the β,ε-carotenoids required in formation of the proximal and distal antennae systems but not in the LHCs.

Key words: β,β- and β,ε-Carotenoid; Chloroplast development; Light-harvesting complex; Lutein; Photosynthetic apparatus; Mutants of *Scenedesmus*

1. Introduction

The pigment composition of the chloroplast of higher plants normally consists of Chls *a* and *b* and the major carotenoids including α- and β-carotene, lutein, zeaxanthin, violaxanthin and neoxanthin [1,2]. In the Chlorophyceae, such as *Scenedesmus obliquus*, an additional carotenoid, loroxanthin, is found in significant amounts. The various carotenoids are distributed among the chlorophyll-protein complexes of the chloroplasts in a diverse pattern with β-carotene predominating in the photosystem reaction centers and the xanthophylls in the proximal and distal antennae systems [3–6] where they fulfill the generally ascribed role of the carotenoids as accessory pigments and anti-photooxidants. In higher plant systems the importance of β-carotene and its derivates is particularly stressed in terms of the carotene associated with the reaction centers and the participation of zeaxanthin, antheraxanthin and violaxanthin in the so-called 'xanthophyll cycle' [1,7].

Recent findings that lutein fulfils a key role in the development of photosystem II activity [8–10], in the reconstitution of LHC II [11–14] and its ubiquitous presence in the LHC of chloroplast from a variety of higher plants [2,15] are important observations which underscore the essential nature of the β,ε-carotenoids in the photosynthetic process of plants possessing both chlorophylls and the LHCs of the two photosystems. With new detergent gel electrophoretic techniques [14] it is now clear that lutein is the dominant carotenoid present in various percentages in the many pigment–protein systems currently identified [4,5,15].

Our recent studies on the adaptation of the normal and LHC-deficient strains of *Scenedesmus obliquus* to low and high intensities of white light showed that preferential changes in the pool sizes of the β,ε-carotenoid occurred reflecting the essential nature of lutein in the development of the LHC-II [16–19]. To obtain additional information about the precise function of specific carotenoids in the photosynthetic apparatus, mutations of the developmental strain of *Scenedesmus*, C-2A′, have been sought in which the β,β-carotenoid and the β,ε-carotenoid pathways would be specifically altered. Evidence will be presented here showing that it is possible to prevent by selective mutation the synthesis of the β,ε-carotenoids without impairing either the biosynthesis of the β,β-carotenoids or the antennae pigment systems directly associated with PS-I and PS-II. However, the loss of the β,ε-carotenoids limits the production of the LHC-II and its associated chlorophylls.

2. Materials and methods

2.1. Organisms, maintenance, growth and greening

The wild type and the secondary mutant strains of C-2A′, C-2A′-34, C-2A′-LHC$_1$ and C-2A′-LHC$_1$, X-1, of *Scenedesmus obliquus* were grown either heterotrophically or mixotrophically at 30°C as originally described [20]. The secondary mutants of C-2A′ were induced by treatment of cells with EMS through established procedures [21]. C-2A′-34 is a newly isolated strain of C-2A′ that possesses specific deletions in the pathway for β,ε-carotenoid biosynthesis. Mutant C-2A-LHC$_1$ [17,18], used for comparison, has no LHC$_1$ and no Chl *b*. The criteria employed in its initial selection were based upon the color of individual clones of cells following ten days of heterotrophic growth at 30°C on agar plates before and after 12 h of illumination in white light (10 Wm^{-2}). The keto carotenoid deficient strain, C-2A′-LHC$_1$, X-1 [18] was used for establishing the various intermediates involved in the biosynthesis of the keto carotenoids in *Scenedesmus*. The procedure employed for irradiation of dark grown cultures was the same as those previously

*Corresponding author.

Abbreviations: Carotenoids: Antheraxanthin = (3*S*,5*R*,6*S*,3′*R*)-5,6-Epoxy-5,6-dihydro-β,β-carotene-3,3′diol; Loroxanthin = (3*R*,3′*R*,6′*R*)-β,ε-Carotene-3,19,3′triol; Lutein = (3*R*,3′*R*,6′*R*)-β,ε-Carotene-3,3′diol; Neoxanthin = (3*S*,5*R*,6*R*,3′8,5′*R*,6′8)-5′,6′-Epoxy-6,7-didehydro-5,6,5′6′-tetrahydro-β,β-Carotene-3,5,3′triol; Violaxanthin = (3*R*,5*R*,6*S*,5′*R*,6′*S*)-5,6,5′,6′-Diepoxy-5,6,5′6′-tetradehydro-β,β- Carotene-3,3′diol; Zeaxanthin = (3*R*,3′*R*)-β,β-carotene-3,3′-diol; Chl *a* = chlorophyll a; Chl *b* = chlorophyll b; EMS = ethyl ester of methylsulfonic acid; LHC = light harvesting complex; PCV = packed cell volume; PPC = pigment–protein complex; PS-I = photosystem I; PS- II = photosystem II; rpHPLC = reversed phase high performance liquid chromatography; WT = normal wild-type of *Scenedesmus*.

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employed [19,20]. PCV was determined with the precautions noted earlier [19].

2.2. Pigment extraction and quantitation

Pigment extraction and rpHPLC quantitation of individual chloroplast pigments were performed by the procedures recently described [19]. Concentrations of individual carotenoids and chlorophylls were determined directly from the HPLC profiles calibrated against standard pigment samples [9]. The peak identified as β-zeacarotene was identified by comparison of our profiles to those obtained in studies on mutant C-6D of *Scenedesmus* [8,9]. Preliminary characterization of the pigment composition of C-2A′-34 was performed with standard thin layer chromatography with either silica gel or a mixture of $Ca(OH)_2$, MgO, and $CaCO_3$ [22] as the adsorbent and hexane/isopropanol/water (100:12:0.5) as the solvent. Plates prepared with the latter adsorbent were used to separate the zeaxanthin-lutein and violaxanthin-loroxanthin bands which did not resolve on silica gel plates. The initial identification of select colored bands separated by TLC as keto carotenoids was made by comparison of TLC plates developed for pigment extracts of whole cells of C-2A′ and the keto carotenoid deficient mutant, C-2A′-LHC-X-1. Individual bands were removed from the TLC plate, eluted with acetone and absorption spectra of individual bands determined in several different solvents. Provisional identification of each keto carotenoid was made by comparison to published literature values

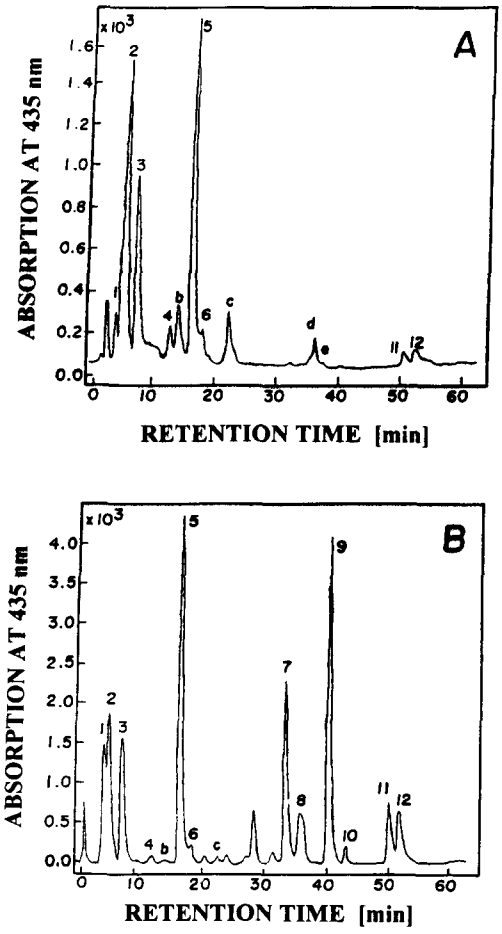

Fig. 1. HPLC elution patterns of the total pigment extracts of dark grown (A) and fully greened (B) cells of C-2A′. The HPLC procedure used was the same as described in [19]. Growth and greening of C-2A′ cells was as indicted in section 2. Identification of numbered (lettered) peaks is as follows: (A) 1, neoxanthin; 2, loroxanthin; 3, violaxanthin; 4, antheraxanthin; 5, lutein; 6, zeaxanthin; 11, β-carotene; 12, α-carotene; a, unknown; b, astaxanthin; c, canthaxanthin; d, echinenone; e, isocryptoxanthin; f, β-zeaxanthin. (B) Peaks 1–6 and 11,12 as in dark sample; 7, Chl *b*; 8, Chl *b*′; 9, Chl *a*; 10, Chl *a*′.

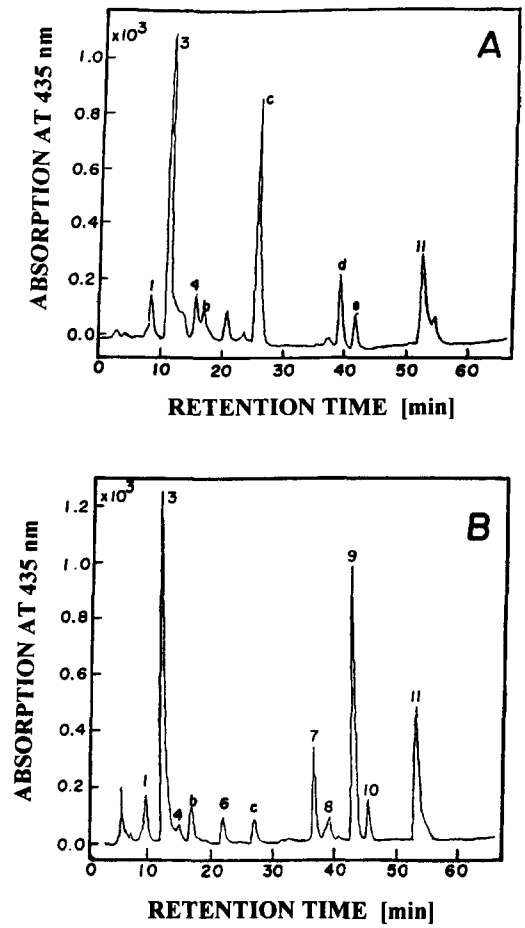

Fig. 2. HPLC elutionof the total pigment extracts of dark grown (A) and fully greend (B) cells of C-2A′-34. Identification of peaks as indicated in legend of Fig. 1.

[1,2]. Concentration of Chls *a* and *b* were determined with the recently developed extinction coeffecients of Porra [23].

2.3. Spectroscopy

Absorption spectra and absorption difference-spectra of whole cells of the various phenotypes used were measured with an Aminco DW-2 spectrophotometer with a PCV of 1 μl/ml.

3. Results

3.1. Pigment composition of mutant strains under different growth conditions

As a preliminary approach in isolating the carotenoid deficient strains, the pigment composition of select mutant isolates was examined by a combination of absorption difference spectra and thin layer chromatographic analyses (data not shown). This procedure allowed for the original selection of the secondary mutant phenotype, C-2A′-34. A more exact comparison of the pigment composition of cells of C-2A′-34 grown in the dark and subsequently exposed to light for 24 h was carried out by HPLC analysis. Representative elution profiles are shown in Figs. 1 and 2 for dark and light grown cells of C-2A′ and C-2A′-34. In Table 1 the quantitative data for each of the major pigments resolved by the procedure are shown for C-2A′ and

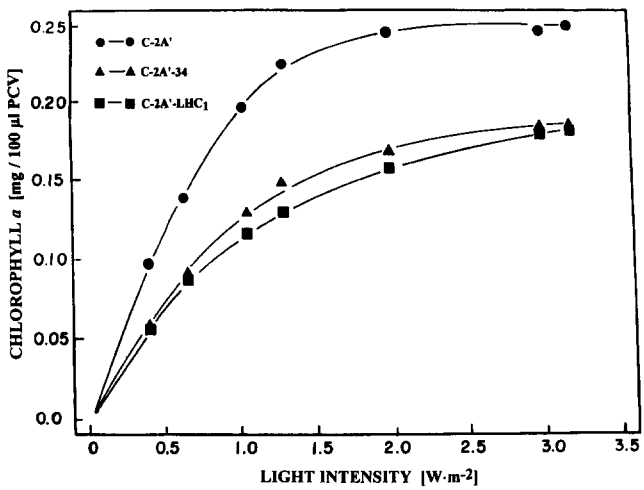

Fig. 3. Light intensity dependency for greening of cells of C-2A′ (●), C-2A′-34 (▲) and C-2A′-LHC$_1$ (■). Cultures were grown heterotrophically for 48 h at 30°C prior to the 24 h of illumination at the indicated intensities. Mutant C-2A′-LHC is characterized by a total Chl *b* and LHC deficiency. The total pigments of 100 µl/ml PCV of cells of each phenotype were extracted and the concentration of Chl *a* determined with the modified procedure outlined in [19].

C-2A′-34. Concomitant with the synthesis of chlorophyll and the development of photosynthetic competence when exposed to light, both strains show increased amounts of their major carotenoids as has been previously noted for *Scenedesmus* [9]. As an apparent compensation for its inability to synthesize the β,ε-carotenoids, C-2A′-34 produces considerably more β-zeacarotene (peak f 01 Fig. 2A), β-carotene (3.4X) and violaxanthin (3.8 X) than does C-2A′. An additional feature of this strain is its inability to develop normal levels of Chl *b* under the growth conditions tested in this study. This feature is reflected in the increase in the Chl *a*/Chl *b* ratio from 3.0 for C-2A′ to 6.0 for C-2A′-34.

Scenedesmus responds to changes in incident light intensities by adapting the relative sizes of the proximal and distal antennae systems [16,19]. Comparison of the pigment content of cells

Table 1
Comparison of the pigment composition of cells of the developmental mutants C-2A′ and C-2A′-34 grown heterotrophically (dark) and when exposed to white light

	C-2A′		C-2A′-34	
	Dark	Light	Dark	Light
Neoxanthin	0.039	0.222	0.021	0.143
Loroxanthin	0.095	0.200	0	0
Violaxanthin	0.088	0.210	0.134	0.800
Lutein	0.114	0.492	0	0
Zeaxanthin	0.013	0.042	0.017	0.067
α-Carotene	0.011	0.146	0	0
β-Carotene	0.016	0.265	0.080	0.893
Chl *b*	0	0.871	0	0.584
Chl *a*	0	2.622	0.222	3.524
Chl*a*/Chl *b*	nd	3.0	nd	6.0

Heterotrophic cultures grown 72 h at 30°C prior to exposure to white light (intensity = 20 W/m^2) for 24 h. Because the mutants are normally unable to synthesize chlorophyll during heterotrophic growth, pigment concentrations are given as µg/µl PCV for this table. nd = not determined.

of C-2A′ and C-2A′-34 grown heterotrophically and subsequently exposed for 24 h to low (4 W/m^{-2}) and high (20 W/m^{-2}) light intensities confirms that the level of the β,ε-carotenoids in C-2A′ decreases at the higher light intensity whereas only minor changes are noted in the β,β-carotenoids (Table 2). Cells of 2A′-34 apparently adapt to low and high intensity light by a somewhat analagous fashion but by utilizing the excess violaxanthin and β-carotene in the development of its proximal and distal antennae systems. We note that the level of Chl *b* in this experiment is decreased by about 50% as was also seen in the data of Table 1. We assume that this feature of C-2A′-34 is a result of the loss of the β,ε-carotenoids, particularly lutein, and not because of an inability to synthesize Chl *b*.

3.2. *Light-intensity dependence of greening in C-2A′ and C-2A′-34*

The observed ability of C-2A′-34 to develop chlorophyll and increased amounts of the β,β-carotenoids when exposed to white light of moderately high intensity (Tables 1 and 2) reveals that the absence of the β,ε-carotenoids in this strain does not result in any short-term light sensitivity. This observation and interpretation is confirmed when the kinetics of Chl *a* production in this mutant and C-2A′ are evaluated over a wider range of light intensity (Fig. 3) wherein it is noted that the kinetics for

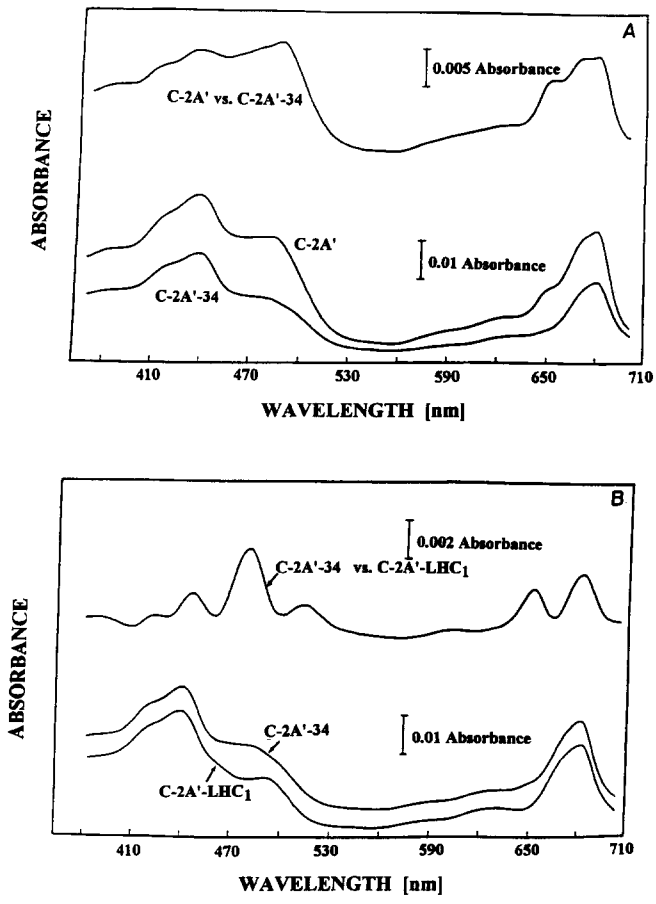

Fig. 4. Room temperature absorption spectra of fully greened, intact cells of C-2A′, C-2A′-34 and C-2A′-LHC$_1$ (A) and their respective absorption difference spectra (B). Following a 24 h greening period the PCV was carefully determined and a suspension of each phenotype containing 1 µl/ml PCV placed in a cuvette of 1 cm pathlength. Mutant C-2A′-LHC$_1$ is a strain lacking the LHC-II and Chl *b* [17].

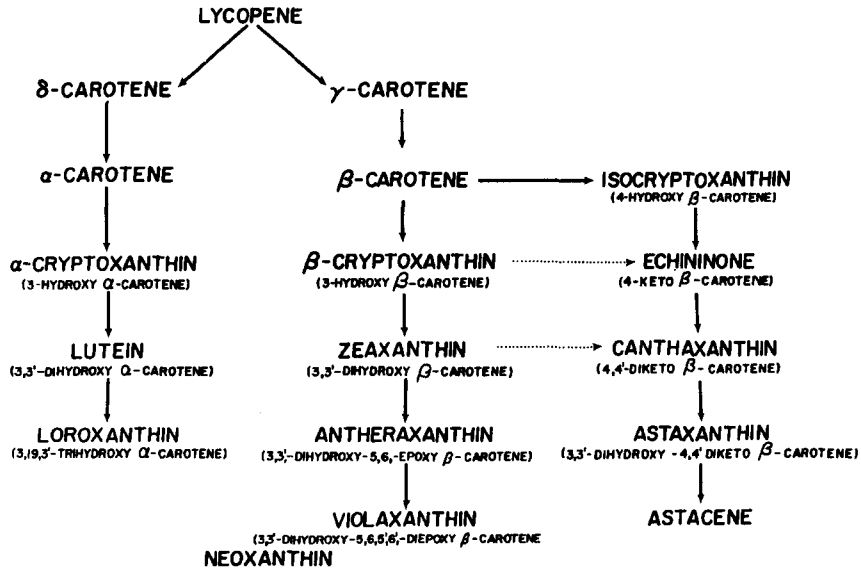

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Fig. 5. Overall scheme for the biosynthesis of the β,ε-, β,β- and keto-carotenoids of *Scenedesmus*. All components in the scheme except for δ-carotene, γ-carotene, α-cryptoxanthin and astacene have been purified and identified by spectral and chromatographic analysis.

greening of C-2A′ and C-2A′-34 are essentially identical. A comparable experiment performed at intensities up to 80 W/m^{-2} also showed no major photoinhibition in either strain (data not presented). Whether C-2A′-34 is stable under prolonged autotrophic growth conditions is still under investigation.

3.3. Absorption and absorption-difference spectra analyses

The absorption spectra and the difference spectra for fully greened cells of C-2A′, C-2A′-34 and C-2A-LHC$_1$ are compared in Fig. 4a and 4b, respectively. The deficiency of LHC-II in 2A′-34 is revealed by the general decrease in absorption resulting from the loss of both chlorophylls and especially for chl *b* in the region between 450 and 500 nm and the loss of the shoulder in the red region of the spectrum (650 nm). The presence of some Chl *b* in C-2A′-34 is also evident by the peaks at 480 and 650 nm in the difference spectrum constructed for C-2A′-34 and C-2A′-LHC$_1$.

We interpret these observations as showing that the absence of the β,ε-carotenes in C-2A′-34 restricts the development of a stable LHC-II.

Table 2
Pigment composition of the developmental mutants of *Scenedesmus*, C-2A′ C-2A′-34 when illuminated for 24 h under low and high light intensities

	C-2A′		C-2A′-34	
	4 W/m^2	20 W/m^2	4 W/m^2	20 W/m^2
Neoxanthin	13.1	12.6	6.4	6.1
Loroxanthin	15.5	11.4	0	0
Violaxanthin	11.8	12.0	36.0	33.8
Lutein	31.9	26.4	0	0
Zeaxanthin	2.1	2.5	3.3	3.0
α-Carotene	14.1	9.3	0	0
β-Carotene	16.6	15.9	52.4	42.2
Chl *b*	35.2	32.9	18.0	15.3
Chl *a*	100.0	100.0	100.0	100.0
Chl *a*/Chl *b*	2.84	3.06	5.56	6.54

Analysis made by standard HPLC procedure [19]. Cultures grown heterotrophically for 72 at 30°C, harvested and defined packed cell volumes extracted in boiling methanol. All concentrations are presented as mol pigment/100 mol Chl *a*. Values are the means of two independent determinations.

4. Discussion

Only three distinct enzymes have been identified in the early pathway for carotenoid biosynthesis. Two of these are involved in the formation of phytoene and its desaturation to the level of lycopene [1,25–29]. The gene for the enzyme responsible for the formation of β-carotene from lycopene, lycopene cyclase, has recently been cloned from *Synechococcus* sp. PCC7942 [30]. Evidence for other enzymatic dependent steps in the formation of the xanthophylls is currently limited. However, our recent mutant studies reveal that at least three additional enzymatic sites in carotenoid biosynthesis can be identified. In Fig. 5 a provisional pathway for total carotenoid biosynthesis is shown for *Scenedesmus*. Mutation sites are shown for lycopene cyclase (C-2A-34), zeaxanthin epoxidase (C-2A′-67), and 'β-carotene 4-oxidase' (C-2A′-LHC,X-1). The phenotypic characteristics of mutant C-2A′-LHC-X-1 have been described [18] and the unique features of the zeaxanthin enriched strain, C-2A′-67, will be presented in a later publication.

The specific loss of β,ε-carotenoid biosynthesis in mutant C-2A′-34, without alteration of the mechanism for β,β-carotenoid biosynthesis, demonstrates for the first time the existence of genetically independent pathways for these two major types of carotenoids. Because it is already known from C^{14}- and tritium-labeled mevalonic acid feeding experiments that the formation of the β- and ε-rings proceed through independent mechanism and that β-rings do not arise directly from ε-rings (reviewed in [7], we propose that two mechanisms for utilizing

lycopene for carotene biosynthesis probably exist in those plants possessing both β,β- and β,ε-carotenoids. Since no build up of potential early intermediates except perhaps β-zeacarotene was observed in pigment extracts of C-2A′-34 (Fig. 2) but increased levels of the β-carotene and violaxanthin were, this supports the concept of two associated, but independent, mechanisms for the initial cyclization step in the cells of the normal WT phenotype. We propose that C-2A′-34 retains only a (β,β)lycopene cyclase and thus funnels all available lycopene to the β,β-carotenoid pathway. It is the additional presence of the (β,ε)lycopene cyclase in plants that results in the production of carotene and its derivates.

An additional unique feature of C-2A′-34 is the associated deficiency in Chl *b* and LHC-II. The absorption and absorption-difference spectra shown in Fig. 3 show clearly that the mutant has decreased levels of both chlorophylls. LDS-PAGE of isolated chloroplast membranes demonstrates a decreased level of the LHC-II even though the amount of LHC polypeptides appears unchanged (data not presented). The absence of lutein in this mutant, a carotenoid which comprises about 60 percent of the total carotenoid pool in a majority of higher plants [1,7], appears to be associated with the development of an unstable light-harvesting complex. This interpretation is supported by the earlier findings that lutein was the most effective carotenoid in reconstituting of LHC from a mixture containing the chlorophylls and the apoproteins of LHC-II [11–14]. We have demonstrated earlier that the inability to synthesize Chl *b* in several LHC-deficient strains, including 2A′LHC$_1$, is not associated with major changes in their carotenoid composition [17,19]. Consequently, it appears that the in vivo assembly of the LHC requires the presence of lutein for maximum binding and stability of the chlorophylls.

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References

[1] Britton, G. (1988) In: Plant Pigments (Goodwin, T.W., Ed.) Chapter 3, pp. 133–182, Academic Press, London.
[2] Rowan, K.S. (1989) Photosynthetic Pigments of Algae, Cambridge University Press, New York.
[3] Siefermann-Harms, D. (1985) Biochim. Biophys. Acta 811, 325–355.
[4] Thornber, J.P. (1986) In: Encyclopedia of Plant Physiology, New Series, Vol. 19, Photosynthesis III, Springer-Verlag Berlin, pp. 98–142.
[5] Bassi, R., Rigoni, F. and Giacometti, G.M. (1990) Photochem. Photobiol. 52, 1187–1206.
[6] Dainese, P., Marquardt, J., Pineau, B. and Bassi, R. (1992) In: Research in Photosynthesis, Vol. I, (Murata, Ed.), Kluwer Academic Publishers, pp. 287–294.
[7] Goodwin, T.W. (1988) In: Plant Pigments, Academic Press, London.
[8] Humbeck, K., Römer, S. and Senger, H. (1988) Botanica Acta 101, 220–228.
[9] Humbeck, K., Römer, S. and Senger, H. (1989) Planta 179, 179–250.
[10] Markgraf, T. and Oelmüller, R. (1991) Planta 185, 97–104.
[11] Plumley, F.G. and Schmidt, G.W. (1987) Proc. Natl. Acad. Sci. USA 84, 146–150.
[12] Cammarata, K., Plumley, F.G. and Schmidt, G.W. (1990) In: Current Research in Photosynthesis (Baltscheffsky, M., Ed) Vol. II, pp. 341–344, Kluwer, Dordrecht.
[13] Paulsen, H., Rumler, U., and Rüdiger, W. (1990) Planta 181, 204–211.
[14] Steinmetz, D., Damm, I. and Grimme L.H. (1990) In: Current Research in Photosynthesis (Baltscheffsky, M. Ed.) Vol. II, pp. 855–858.
[15] Peter, G.F. and Thornber, J.P. (1951) J. Biol. Chem. 166, 16745–16754.
[16] Bishop, N.I., Humbeck, K., Römer, S. and Senger, H. (1989) J. Plant Physiol. 135, 144–149.
[17] Bishop, N.I. and Maggard, S. (1992) In: Regulation of Chloroplast Biogenesis, (Argyroudi-Akoyunoglou, J.H. Ed.) pp. 603–618, Plenum Press.
[18] Bishop, N.I., Hermsmeier, D. and Senger, H. (1992) In: Research in Photosynthesis (Murata, N. ed.) Vol. III, pp. 433–436, Kluwer, Dordrecht.
[19] Senger, H., Wagner, C., Hermsmeier, D., Hohl, N., Urbig, T., and Bishop, N.I. (1993) J. Photochem. Photobiol. B: Biol. 18, 273–279.
[20] Senger, H. and Bishop, N.I. (1972) Plant Cell Physiol. 13, 633–649.
[21] Bishop, N.I. (1982) In: Methods in Chloroplast Molecular Biology (Edelman, M., Hallick, R.B. Chua, N.-H. Eds) pp. 51–64, Elsevier Biomedical Press, New York.
[22] Weber, A. and Czygan, F.-C. (1972) Arch. Mikrobiol. 84, 243–253.
[23] Porra, R.J. (1991) In: Chlorophylls (Scheer, H. Ed) pp. 31–57, CRC Press, Boca Raton.
[24] Humbeck, K. and Bishop, N.I. (1986) Plant Cell Physiol. 27, 1407–1417.
[25] Bartley, E.B. and Scolnik, P.A. (1994) Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 287–301.
[26] Dogbo, D., Laferriere, A., D'Harlingue, A. and Camara, B. (1985) Proc. Natl. Acad. Sci. USA 85, 7054–7058.
[27] Heide, L. and Berger, U. (1989) Arch. Biochem. Biophys. 273, 331–338.
[28] Sandmann, G. and Kowalezykd, S. (1939) Biochem. Biophys. Res. Commun. 163, 916–921.
[29] Hugueney, P., Romar, S., Kuntz, M. and Camara, B. (1992) Eur. J. Biochem. 209, 399–407.
[30] Cunningham, F.X., Chamovitz, D., Misawa, N., Gantt, E., and Hirschberg, J. (1993) FEBS Lett. 328, 130–138.