FEBS Letters 580 (2006) 4718–4722

Elucidation of the β-carotene hydroxylation pathway in *Arabidopsis thaliana*

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Received 14 June 2006; revised 13 July 2006; accepted 14 July 2006

Available online 26 July 2006

Edited by Richard Cogdell

Abstract The first dedicated step in plant xanthophyll biosynthesis is carotenoid hydroxylation. In *Arabidopsis thaliana*, this reaction is performed by both heme (LUT1 and LUT5) and non-heme (CHY1 and CHY2) hydroxylases. No mutant completely abolishing α- or β-carotene hydroxylation has been described to date. We constructed double and triple mutant combinations in *CHY1*, *CHY2*, *LUT1*, *LUT5* and *LUT2* (lycopene epsilon-cyclase). In *chy1chy2lut2*, 80% of leaf carotenoids is represented by β-carotene. In *chy1chy2lut5*, β-carotene hydroxylation is completely abolished, while hydroxylation of the β-ring of α-carotene is still observed. The data are consistent with a role of LUT5 in β-ring hydroxylation, and with the existence of an additional hydroxylase, acting on the β-ring of α-, but not β-carotene.

Keywords: Carotenoid biosynthesis; Carotene hydroxylases; Xanthophylls; *Arabidopsis*

1. Introduction

Xanthophylls are oxygenated carotenoids that play a pivotal role in the photosynthetic apparatus of higher plants. The first dedicated step in plant xanthophyll biosynthesis is the hydroxylation of the ε- and β-ionone rings of α-carotene (ε–β-carotene) and β-carotene (β–β-carotene) through the insertion of iron-activated oxygen (Fig. 1).

Two different classes of enzymes are involved in these reactions: Ferredoxin-dependent diiron oxygenases (CHY1 and CHY2), active in β-ring hydroxylation [1–3], and cytochromes P450: LUT1/CYP97c1, required for ε-ring hydroxylation [4] and LUT5/CYP97a3, showing in vivo a major hydroxylation activity on the β-ring of α-carotene and a minor activity on the β-rings of β-carotene [5]. In *Arabidopsis*, the double *chy1-chy2* mutant shows greatly reduced levels of xanthophylls containing two hydroxylated β-rings (β–β-xanthophylls); introduction of the *lut1* mutation in the *chy1chy2* background further reduces, but does not completely eliminate, β-β-xanthophylls [3]. This observation suggests that yet another hydroxylase must be active in the synthesis of β–β-xanthophylls. This hydroxylase is probably also active in the hydroxylation of the single β-ring of α-carotene, since this reaction is largely unaffected in the *chy1chy2* and *lut1* mutants. A candidate for this missing β-hydroxylase activity is LUT5 [4,5]. *lut1* mutants lack lutein and accumulate its β-monohydroxylated precursor, zeinoxanthin [6,4] suggesting that, during lutein biosynthesis, hydroxylation of the β-ring occurs independently from that of the ε-ring.

Due to the redundancy of plant carotenoid hydroxylases, no mutant completely arrested in hydroxylation of either α- or β-carotene has been reported to date. To obtain such a mutant, we have isolated T-DNA insertion mutants in xanthophyll biosynthesis genes, selected double and triple mutant combinations, and analyzed their biochemical phenotypes.

2. Materials and methods

T-DNA insertion mutants were identified in the Syngenta [7] and Salk [8] collections. All mutants were in the Columbia background and were grown at 22 °C under a photoperiod of 16 h light (30 μE m^{-2} s^{-1}) and 8 h darkness. Individual mutants were crossed, and F1 seeds were grown and self-fertilized to obtain the F2 generation. The genotype of the F2 individuals was checked by PCR using gene-specific primers and T-DNA primers (Table 1).

RNA extraction and semi-quantitative RT-PCR were performed as described [9], with modifications [10]. Oligonucleotides localized on exon sequences, flanking the insertion site, were used for the PCR step (Table 1).

HPLC separation and quantification of pigments were performed using two different chromatographic systems.

In system 1, leaf samples were frozen in liquid nitrogen, ground in 85% acetone buffered with Na_2CO_3, then the supernatant of each sample was recovered after centrifugation (15′ at 15000 × *g*, 4 °C); quantification of pigments was performed using a System Gold HPLC-PDA (Beckman) apparatus and published chromatographic methods [11] and by fitting of the spectrum of the acetone extract with spectra of individual pigments [12]. For methylation of allylic hydroxyl groups [13], peaks were collected using a Biorad 2110 fraction collector, vacuum dried, the pellet was dissolved in 5 ml methanol, then few drops of HCl (0.2 N) were added. After 2 h at RT, carotenoids were dried, resuspended in diethyl ether and re-chromatographed as described above.

In system 2, leaf material was freeze-dried, ground into powder, extracted with methanol/water (1:1) (500 μl/10 mg powder). In order to remove chlorophyll, saponification was performed with KOH (final concentration 6% w/v) at 50 °C in the dark for 1 h. The mixture was extracted twice with 1 ml chloroform, centrifuged, and the resulting hypophases were pooled, dried under a stream of nitrogen, re-dissolved in ethyl acetate (HPLC grade, VWR Poole, UK). Canthaxanthin (2 μg) was added, the extract was centrifuged, injected onto a

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0014-5793/$32.00
doi:10.1016/j.febslet.2006.07.055

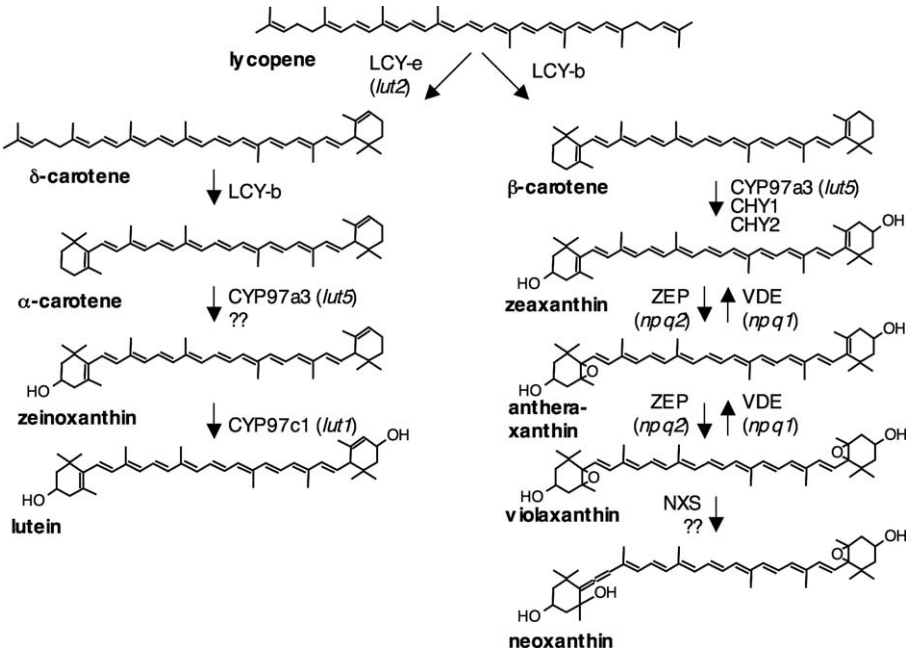

Fig. 1. Carotenoid biosynthesis pathway in *Arabidopsis thaliana* leaves. The names of the enzymes controlling each step are indicated. When different, the mutant names are given in parentheses.

Table 1
Oligonucleotides used sequences of oligonucleotides used for PCR confirmation of the insertions or for RT-PCR measurement of transcripts

PCR	
T-DNA (Syngenta)	TAGCATCTGAATTTCATAACCAAT
T-DNA (Salk)	TGGTTCACGTAGTGGGCCATCG
CHY1 forward	CATTCAAACCACTCCACCGC
CHY1 reverse	TCGTTTAGCTCAAACGGTC
CHY2 forward	GCCATCACCTCTCCTTACCT
CHY2 reverse	GCCCAAAACTCCATCCCAA
LUT2 forward	ACTATCTTCATGGTCTGTG
LUT2 reverse	TCGTAAGTCTTTAGAATTCG
LUT1 forward	TGATTGTGGAGAGAGTATTC
LUT1 reverse	GAATGTCAGGAACTTGAGC
LUT5 forward	CGTACTCTCGGAGATCGAAC
LUT5 reverse	TATCAAGTGTCAAACGAGAG
RT-PCR	
CHY1 forward	CATAGAGCTCTGTGGCACGC
CHY1 reverse	AGGGACGTCGGCGATGGGAC
CHY2 forward	AGCAGGACTATCAACAATCG
CHY2 reverse	GCCCAAAACTCCATCCCAA
LUT2 forward	GTATTGAGCATGTTTGGAG
LUT2 reverse	TCGTAAGTCTTTAGAATTCG
LUT1 forward	TGATTGTGGAGAGAGTATTC
LUT1 reverse	GAATGTCAGGAACTTGAGC
LUT5 forward	CGTACTCTCGGAGATCGAAC
LUT5 reverse	TATCAAGTGTCAAACGAGAG
ACTIN forward	TGGCGATGAAGCTCAATCC
ACTIN reverse	CACTGGCATAAAGAGAAAG

reverse-phase C_{30} column (YMC, Inc., Wilmington, NC, USA) and developed using a methanol, *tert*-methyl butyl ether gradient system and a Waters Alliance HPLC system with an on-line photo diode array detector [14]. Authentic standards of α-carotene, β-carotene, and δ-carotene were prepared from carrots, ripe wild type tomato and *Del,-Del* mutant tomato fruit [14].

MALDI-TOF/MS: The α-carotene peak was collected and extracted from the mobile phase of chromatographic system 2 by first evaporating the *tert*-methyl butyl ether present in the sample with a stream of nitrogen gas. One volume of dH_2O and two of chloroform were added, the mixture was centrifuged at 3500 rpm for 3 min and the hypophase was collected and evaporated under nitrogen. Carotenoid samples were re-dissolved in ethyl acetate (ca. 1 μg/μl), 1 μl of carotenoid sample was mixed with 1 μl of matrix (β-cyan-4-hydroycinnamic acid) and an aliquot (0.5–1 μl) spotted on a MALDI target plate (Bruker Daltonics) and allowed to air-dry. MALDI-TOF spectra were acquired with a Bruker MALDI-TOF Reflex III (Coventry, UK) operated in the positive reflectron mode, set at a source voltage of 25 kV and extraction voltage of 20.7 kV with no ion suppression. Scans were extended to 3000 *m/z* initially and data acquired with 15 shots/scan and 3 scans/spectrum. The mass spectrometer was calibrated with authentic carotenoid standards.

3. Results and discussion

3.1. Construction of double and triple mutants

T-DNA insertion mutants in the genes encoding the two non-heme hydroxylases (*CHY1* and *CHY2*), the ε-ring hydroxylase (*LUT1*), its paralog (*LUT5*) and the ε-cyclase (*LUT2*) were obtained from Syngenta and the Salk institute (Fig. 2). All the T-DNA insertions, with the exception of *chy1*, disrupted exon sequences within the first half of the protein (Fig. 2), thus they probably represent functional knock-outs. All the insertions segregated as single Mendelian loci and contained greatly reduced or absent levels of transcripts for the mutated genes (Fig. 3). The mutants were crossed with each other under low light conditions (30 $\mu E\, m^{-2}\, s^{-1}$) and the inheritance of the insertions as well as their presence in homozygous or heterozygous form was followed via PCR using appropriate oligonucleotides (Table 1). None of the single or double mutants displayed a visible phenotype, while the triple mutants *chy1chy2lut2* and *chy1chy2lut5* showed, respectively, paler leaves and a highly retarded growth (Fig. 4).

The leaf carotenoid content of the wild-type and the various mutants was analyzed through diode array HPLC (Table 2). We tested dark-adapted plants (a condition in which the *Wt*

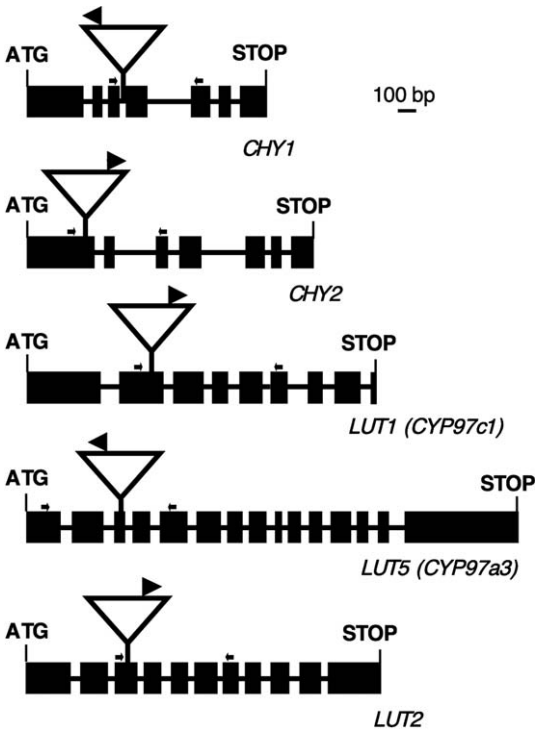

Fig. 2. Gene maps, indicating the site of insertion of the T-DNA. Boxes represent exons, thin lines represent introns. Arrowheads on the T-DNAs indicate the orientation of the oligonucleotide used for the screening (see Section 2). Arrows on the exons indicate primers used for RT-PCR (see Table 1). The lines used are: *CHY1*: SAIL line 49A07; *CHY2*: SAIL line 1242B12; *LUT1*: Salk line 129724; *LUT2*: Salk line 005018; *LUT5*: Salk line 116660.

accumulates violaxanthin as the main β–β-xanthophyll) and plants transferred for 45′ under high light conditions (1200 μE m^{-2} s^{-1}) a condition in which violaxanthin is de-epoxidated into zeaxanthin [15]. In agreement with previous results [3], the *chy1chy2* double mutant showed reduced conversion of β-carotene into β–β-xanthophylls. The triple *chy1-chy2lut2* mutant additionally lacked lutein, so almost 80% of its leaf carotenoids were represented by β-carotene (Table 2). In spite of the profound alteration in carotenoid content, this mutant was viable under laboratory conditions.

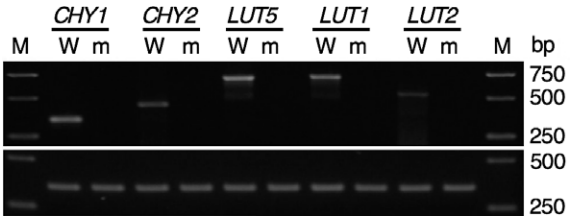

Fig. 3. Semi-quantitative RT-PCR measurement of gene-specific transcripts. Sequences of the oligonucleotides used are shown in Table 1. Top panel: for each gene, RNA extracted from *Wt* (W) and the corresponding mutant (m) was subjected to reverse transcription, followed by 30 cycles of PCR amplification. Bottom panel: amplification of the actin control transcript from the same RNAs. M: molecular weight marker. The expected sizes of the PCR products are: *CHY1*: 273 bp; *CHY2*: 447 bp; *LUT1*: 723 bp; *LUT5*: 705 bp; *LUT2*: 580 bp *ACTIN*: 275 bp.

3.2. The α- and β-carotene hydroxylation pathways

In agreement with previous data [5] the *lut5* mutant shows a reduction of β–β-xanthophylls (violaxanthin, neoxanthin and, under high light conditions, zeaxanthin) and accumulation of α-carotene, a lutein precursor normally not found in *Wt* plants (Table 2). The identity of α-carotene was confirmed by its co-migration with authentic standards (Fig. 5) and by its on-line absorption spectrum (data not shown). MALDI-TOF mass spectrometry of α-carotene confirmed an *m/z* value of 536.34, in agreement with its formula ($C_{40}H_{56}$, data not shown).

These data suggest that LUT5 is involved in β-ring hydroxylation of both α- and β-carotene. β-carotene is reduced in this mutant (Table 2), probably due to the fact that α-carotene competes, on protein-pigment complexes, for the binding sites normally occupied by β-carotene [5]. The accumulation of α-carotene and lutein, but not of α-cryptoxanthin (α-carotene hydroxylated on the ε-ring) suggests that in vivo the preferred substrate for the ε-hydroxylase LUT1 is not α-carotene, but its monohydroxylated derivative, zeinoxanthin.

The *lut1lut5* double mutant accumulates α-carotene and zeinoxanthin, suggesting that at least one additional hydroxylase activity, hydroxylating the β-ring of α-carotene, is still present in this mutant.

The triple mutant *chy1chy2lut5* contains no trace of β-carotene-derived xanthophylls (Fig. 6 and Table 2). The sensitivity of the detection system and the dual condition (dark adaptation and exposure to high light) used, would detect any of these

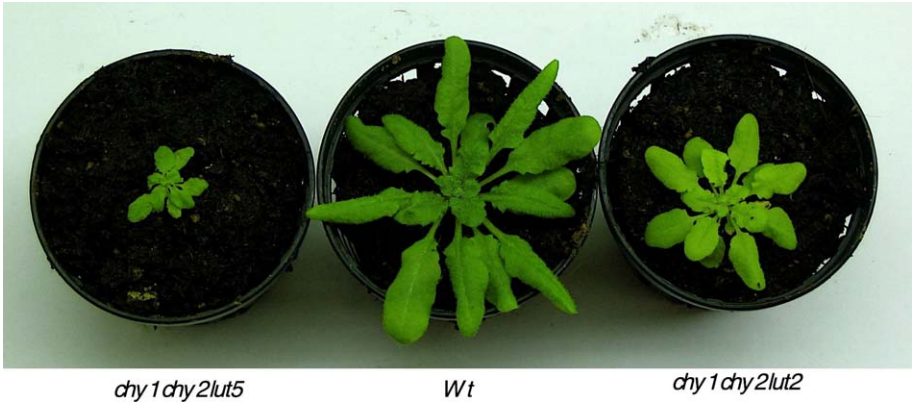

Fig. 4. Phenotypes of 35-days-old *Wt* and mutant plants, grown at 30 μE m^{-2} s^{-1}.

Table 2
HPLC analysis of leaf carotenoid content (mol/100 mol Chl) in dark-adapted and light-treated plants

		β-Car	Zea	Anthera	Viola	Neo	α-Car	Zeino	Lute
Dark-adapted	*Wt*	10.6 ± 0.3	nd	nd	3.4 ± 0.2	4.6 ± 0.1	nd	nd	10.7 ± 0.2
	lut5	6.0 ± 0.4	nd	nd	2.3 ± 0.1	3.3 ± 0.3	8.7 ± 0.2	nd	8.5 ± 0.2
	lut1	9.7 ± 0.5	1.1 ± 0.3	2.0 ± 0.1	5.7 ± 0.2	4.0 ± 0.1	nd	6.9 ± 0.1	nd
	lut2	13.3 ± 0.2	0.7 ± 0.1	1.8 ± 0.1	8.5 ± 0.1	4.2 ± 0.2	nd	nd	nd
	chy1chy2	12.2 ± 0.1	nd	nd	1.4 ± 0.1	1.1 ± 0.2	nd	nd	12.4 ± 0.2
	lut1lut5	6.6 ± 0.5	1.0 ± 0.1	1.7 ± 0.1	3.4 ± 0.1	3.1 ± 0.1	7.7 ± 0.4	5.1 ± 0.2	nd
	chy1chy2lut2	22.3 ± 0.3	1.6 ± 0.1	2.1 ± 0.3	2.6 ± 0.2	1.7 ± 0.2	nd	nd	nd
	chy1chy2lut5	8.4 ± 1.3	nd	nd	nd	nd	11.9 ± 0.4	nd	7.4 ± 1.1
Light-treated	*Wt*	10.4 ± 0.3	2.4 ± 0.3	0.3 ± 0.2	1.3 ± 0.1	4.3 ± 0.1	nd	nd	10.9 ± 0.2
	lut5	5.6 ± 0.1	1.3 ± 0.1	0.4 ± 0.1	0.9 ± 0.1	3.8 ± 0.1	8.3 ± 0.1	nd	8.5 ± 0.1
	lut1	10.1 ± 0.2	4.1 ± 0.1	1.8 ± 0.2	2.3 ± 0.1	3.7 ± 0.2	nd	7.0 ± 0.1	nd
	lut2	12.5 ± 0.2	6.6 ± 0.2	2.2 ± 0.1	3.1 ± 0.1	4.2 ± 0.1	nd	nd	nd
	chy1chy2	12.0 ± 0.1	0.8 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	1.2 ± 0.2	nd	nd	12.4 ± 0.2
	lut1 lut5	6.9 ± 0.1	2.1 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	3.2 ± 0.2	7.3 ± 0.3	4.9 ± 0.1	nd
	chy1chy2lut2	21.8 ± 0.1	3.9 ± 0.2	1.4 ± 0.1	1.1 ± 0.1	2.1 ± 0.2	nd	nd	nd
	chy1chy2lut5	10.1 ± 0.8	nd	nd	nd	nd	9.6 ± 0.2	nd	8.1 ± 0.9

Plants grown at 30 μE m^{-2} s^{-1} for 35 days were dark-adapted for 2 h or treated for 45′ with high intensity light (1200 μE m^{-2} s^{-1} 45′, RT). Carotenoids were extracted and quantified via diode array HPLC using chromatographic system 1 (see Section 2). Data are the average of at least three independent measurements.
nd = not detectable.

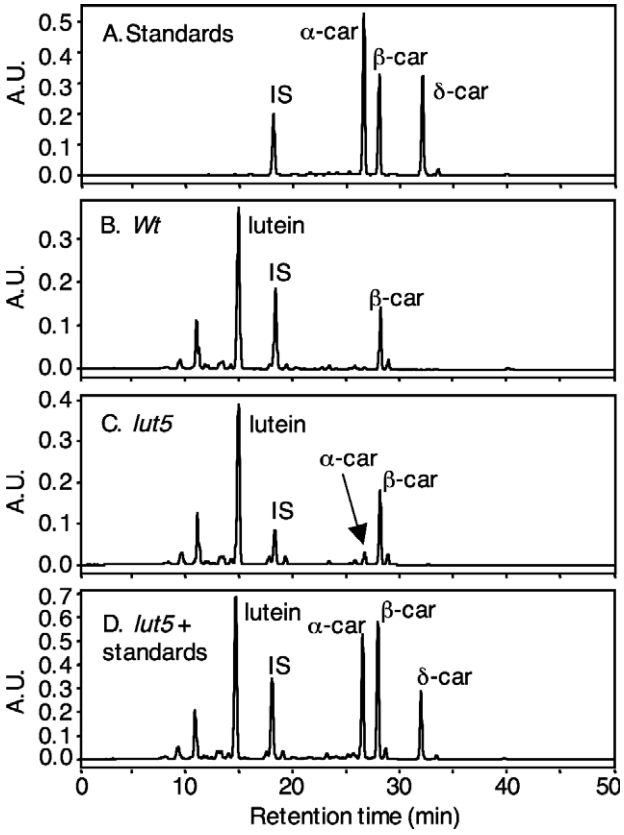

Fig. 5. HPLC identification of carotenoids in the *lut5* mutant. (A) Carotene standards; (B) *Wt* extract; (C) *lut5* extract; (D) *lut5* extract spiked with standards. Carotenoids were separated using chromatographic system 2 (see Section 2). IS: canthaxanthin internal standard. The absorbance at $\lambda = 450$ nm is shown. The identities of the compounds were confirmed by their on-line absorption spectra.

xanthophylls at levels equal or above 3% of wild-type levels. Thus, we conclude that, in our experimental conditions, more than 97% of β-carotene hydroxylation is performed by the CHY1, CHY2 and LUT5 hydroxylases.

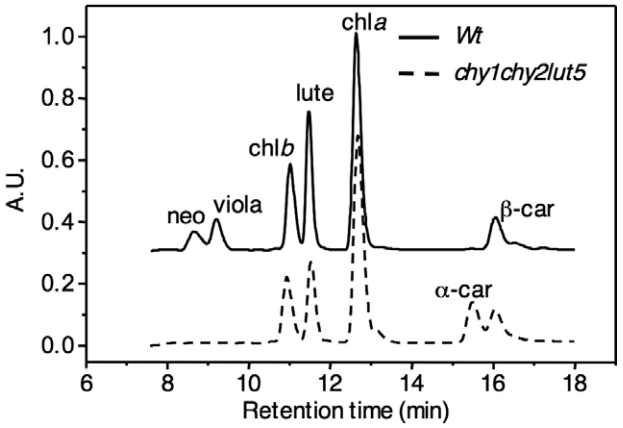

Fig. 6. HPLC identification of carotenoids in the *chy1chy2lut5* mutant. The absorbance at $\lambda = 440$ nm is shown. Carotenoids were separated using chromatographic system 1 (see Section 2). The absorbance at $\lambda = 440$ nm is shown. The identities of the compounds were confirmed by their on-line absorption spectra.

The triple mutant contained high levels of lutein, indicating that at least one additional hydroxylase activity, able to efficiently hydroxylate the β-ring of α-carotene, but not of β-carotene, is present in this mutant.

The identities of lutein from *chy1chy2lut5* and of zeinoxanthin from *lut1lut5* were confirmed by their on-line absorption spectra (data not shown) and by allylic methylation [13]. In this reaction, allylic hydroxyl groups (i.e. that on the ε-ring, but not that on the β-ring) are methylated, changing the carotenoid's retention time. The results (Fig. 7) show that the lutein peak from *chy1chy2lut5* contained, as expected, one allylic hydroxyl group, while the zeinoxanthin peak from *lut1lut5* contained, to the limit of detection of the experiment, no allylic hydroxyl group, and thus no contaminating α-cryptoxanthin.

The most likely explanation of the above findings is that the preferred substrate of LUT1 is zeinoxanthin, and that the β-ring of α-carotene is promiscuously hydroxylated by all four hydroxylases. We are attempting the construction of a

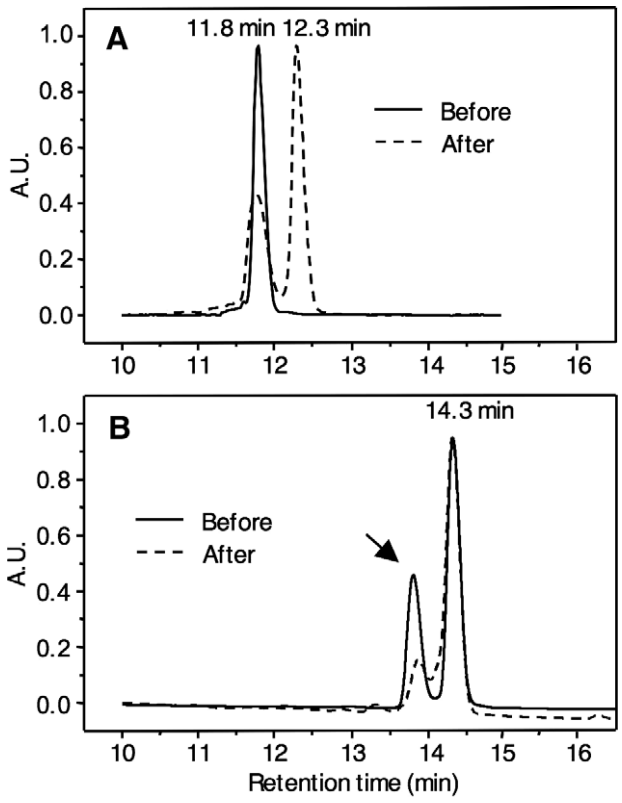

Fig. 7. Allylic methylation. The lutein peak from *chy1chy2lut5* (A) and the zeinoxanthin peak from *lut1lut5* (B) were subjected to allylic methylation (see Section 2) and re-chromatographed using system 1. Retention times were determined before (solid lines) and after (dashed lines) methylation of allylic hydroxyl groups. In panel B, the arrow indicates a contaminating Chl *a* peak, which is partially hydrolyzed under the conditions used for allylic methylation. The absorbance at $\lambda = 470$ nm is shown. The identities of the compounds were confirmed by their on-line absorption spectra.

quadruple *chy1chy2lut1lut5* mutant to clarify this point. However, since this mutant is expected to completely lack xanthophylls, and given the sub-lethal phenotype of *chy1chy2lut5*, the question of the viability of this quadruple mutant remains open. Thus, the plant pathway for α-carotene hydroxylation still awaits complete elucidation.

Acknowledgements: We thank Syngenta and the Salk Institute for providing the *Arabidopsis* insertion lines used in this study. Work supported by the Italian Ministry of Research, Special Fund for Basic Research. A.F. acknowledges the supervision of Prof. Laura Spano' Univ. of L'Aquila, for her doctoral work.

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