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Functional integration of non-native carotenoids into chloroplasts by viral-derived expression of capsanthin–capsorubin synthase in *Nicotiana benthamiana*

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Summary

The biosynthesis of leaf carotenoids in *Nicotiana benthamiana* was altered by forced re-routing of the pathway to the synthesis of capsanthin, a non-native chromoplast-specific xanthophyll, using an RNA viral vector containing capsanthin–capsorubin synthase (*Ccs*) cDNA. The cDNA encoding *Ccs* was placed under the transcriptional control of a tobamovirus subgenomic promoter. Leaves from transfected plants expressing *Ccs* developed an orange phenotype and accumulated high levels of capsanthin (up to 36% of total carotenoids). This phenomenon was associated with thylakoid membrane distortion and reduction of grana stacking. In contrast to the situation prevailing in chromoplasts, capsanthin was not esterified and its increased level was balanced by a concomitant decrease of the major leaf xanthophylls, suggesting an autoregulatory control of chloroplast carotenoid composition. Capsanthin was exclusively recruited into the trimeric and monomeric light-harvesting complexes of photosystem II (PSII) and shown to significantly contribute to the light-harvesting capacity. On a chlorophyll basis, the concentrations of PSI and PSII reaction centres were not modified. This demonstration that higher plant antenna complexes can accommodate non-native carotenoids provides compelling evidence for functional remodelling of photosynthetic membranes toward a better photoreactivity by rational design of the incorporated carotenoid structures.

Introduction

Carotenoids are intrinsic constituents of the photosynthetic apparatus, acting as antenna pigments as well as photoprotectants. Although more than 600 carotenoids have been identified (Britton, 1995), the number of carotenoid structures that could be functionally integrated into photosynthetic membranes is not known. As a consequence of light limitation in aquatic milieu, extreme examples of carotenoid molecule recruitment are frequent in lower phototrophs, prokaryotes and algae which display a positive correlation between the variability of photosynthetic membrane architecture (Drews and Oelze, 1981; Kirk, 1978) and the multiplicity of carotenoid structures (Green and Durnford, 1996; Vechtel *et al.*, 1992; Young, 1993). In contrast, chloroplast thylakoids from higher plants only contain a limited series of ε- and β-cyclohexenyl carotenoids (Young, 1993). An exception to this basic phenomenon has been described for some plant species belonging to the tribe of compositae, where lactucaxanthin, an unusual ε carotenoid homologous to lutein (Siefermann-Harms *et al.*, 1981), is apparently integrated into the light-harvesting complex of photosystem II (LHCII) (Phillip and Young, 1995; Siefermann-Harms and Ninnemann, 1982).

In a more general context, significant accumulation of acyclic carotenoids in plant chloroplasts has been described following inhibitor treatments (Seyama and Splittstoesser, 1975) and mutations (Faludi-Daniel, 1975; Faludi-Daniel *et al.*, 1968). However, these changes are lethal due to pleiotropic effects associated with premature inhibition of the carotenogenic pathway. Thus, manipulation of the later step of the chloroplast carotenogenic pathway, i.e. at the level of xanthophylls, could be more instructive for delineating stringent structural requirements. In addition to their antenna role and their involvement in excess energy dissipation, xanthophylls are required for pigment–protein complex assembly (Paulsen *et al.*, 1990; Plumley and Schmidt, 1987). It has been shown that the dissipation of excess energy through chlorophyll fluorescence quenching in LHCII is strictly dependent upon the length of the xanthophyll chromophores, and those having more than 10 conjugated double bonds offer better efficiency (Phillip *et al.*, 1996). Among usual leaf xanthophylls, this criterion could only be partly fulfilled by zeaxanthin (Frank *et al.*, 1994) which accumulates transiently in leaves subjected to high light stress (Demmig-Adams *et al.*, 1996). Based on these considerations, the availability of new carotenogenic genes offers the opportunity to introduce into leaf chloroplasts non-native xanthophyll molecules that have potentially better photodynamic properties due to their chemical structure.

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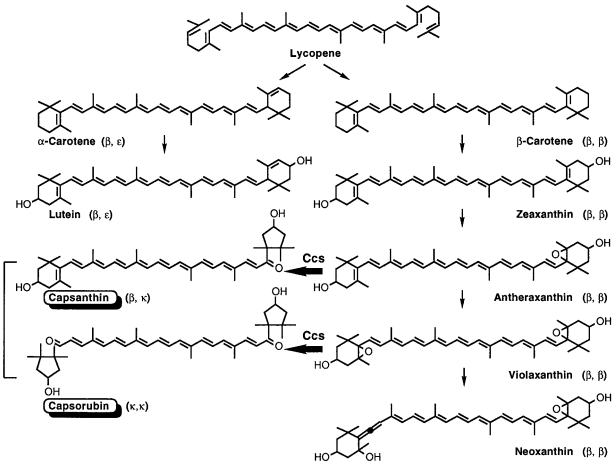

Figure 1. General chloroplast carotenoid and keto-xanthophyll branch pathways.
The general pathway involves the conversion of lycopene to the final products lutein (ε xanthophyll) and neoxanthin (β xanthophyll). Ectopic expression of non-native capsanthin–capsorubin synthase (Ccs) directs the pathway to the formation of capsanthin and capsorubin (κ xanthophyll) through the intermediates antheraxanthin and violaxanthin. The configuration of carotenoid rings from left to right is indicated by Greek letters.

Previously, we cloned the pepper fruit cDNA encoding capsanthin–capsorubin synthase (Ccs), which catalyses the conversion of ubiquitous epoxy-xanthophylls, antheraxanthin and violaxanthin, into the red pigmented chromoplast keto-xanthophylls, capsanthin and capsorubin (Bouvier *et al.*, 1994) (Figure 1). These keto-carotenoids have an extended chromophore of 11 double bonds rendering them more photoactive (Di Mascio *et al.*, 1989; Frank *et al.*, 1994; Nielsen *et al.*, 1996) but they have not been previously introduced into chloroplast membranes. The promoter of the cauliflower mosaic virus 35S generally used to introduce foreign gene in plants results in variable levels of expression (Hobbs *et al.*, 1990) and requires a large sampling to isolate lines in which the transgene is highly expressed. Previous studies using an RNA viral vector demonstrated that the chloroplast carotenoid pathway could be deliberately manipulated (Kumagai *et al.*, 1995). Based on this fact, we decided to use the viral-based system to enable the *de novo* expression of Ccs in *N. benthamiana* leaves and analyse the transfected plants for structural and biochemical alterations. First, alteration of chloroplast carotenogenesis to form capsanthin concomitantly decreased the concentration of native leaf xanthophylls. Second, we provide evidence that capsanthin is exclusively incorporated into light-harvesting complexes of photosystem II and contributes to the light-harvesting capacity. Finally, on a chlorophyll basis, the concentration of photosystem I and II reaction centres remained constant in control and transfected plants, thus revealing a tight coordination of the photosynthetic apparatus assembly.

Results

Phenotypic changes following Ccs *expression in transfected leaves*

In order to manipulate the formation of xanthophylls during carotenoid biosynthesis, the open reading frame (ORF) for *Ccs* was placed under the control of the tobacco mosaic virus (TMV-U1) coat protein subgenomic promoter in both the sense (*Ccs*$^+$) and antisense (*Ccs*$^-$) orientations (Figure 2a). Infectious RNAs from TTO1A*Ccs*$^+$ and TTO1A*Ccs*$^-$ were prepared *in vitro* using SP6 DNA-dependent RNA polymerase and used to mechanically inoculate *N. benthamiana*.

The hybrid viruses spread throughout the non-inoculated upper leaves, as verified by local lesion infectivity assay, electron microscopy (results not shown), and PCR amplification (Figure 2b). The transfected plants accumulated recombinant virions similar in size and yield to wild-type TMV in infected tissue (results not shown). The viral symptoms consisted of the systemic distortion of leaves, plant stunting and mild chlorosis (Figure 3a–c). In plants transfected with *Ccs*$^+$, we observed the development of orange patches which are phenotypically characteristic of variegated plants (Figure 3a–c). The latter phenotype may be causally related to plastid differentiation. Therefore, we decided to further analyse the biochemical and ultrastuctural changes underlying this phenotype.

Synthesis of capsanthin in chloroplasts and modulation of thylakoid membrane morphogenesis

Thin-layer chromatography analysis of the unsaponified lipid extract revealed that a new orange carotenoid, capsanthin, accumulated (Rf 0.45) and was found only in plants transfected with *Ccs*$^+$ (Figure 3d). The capsanthin from virus-infected plants was purified by HPLC and had a unique absorption peak in ethanol with a λ_{max} value at 474 nm or λ_{max} values at –485 and 518 nm in benzene (results not shown). This novel carotenoid had an identical retention time to an authentic capsanthin standard and its migration or elution time was not changed by saponification. After sodium borohydride reduction, the ethanolic solution capsanthin had a spectrum with λ_{max} values at 428, 451 and 479 nm, indicative of keto group reduction and subsequent restoration of the spectral fine structure (results not shown). In chloroplasts expressing sense *Ccs*$^+$, capsanthin is unesterified, a situation which contrasts with chromoplasts where capsanthin is almost totally esterified by fatty acids (Camara and Monéger, 1978).

Leaves from plants transfected with vector control, sense and antisense *Ccs* constructs showed a slight reduction in the chlorophyll content (Table 1). This phenomenon is inherent to the expression system (Kumagai *et al.*, 1995) because

the virus causes mild chlorosis. Although the overall level of carotenoids did not change, there was a dramatic increase in the synthesis and accumulation of capsanthin. Approximately 2 weeks after transfection, leaves from systemically infected TTO1A*Ccs*$^{+}$ plants developed a deep orange phenotype. This colour change can be a useful marker in the analysis of viral insert stability, replication and movement. HPLC analysis revealed that 36% of the total carotenoids (Table 1) were converted to capsanthin (Figure 1). Immunoblot analysis revealed that a 50 kDa protein accumulated in the upper leaves of transfected plants (12 days after inoculation) (Figure 4). No detectable cross-reacting proteins were observed in the non-infected *N. benthamiana* control extracts or in plants transfected with the antisense construct (*Ccs*$^{-}$). The mature Ccs from *C. annuum* has an approximate molecular mass of 50 kDa and is initially synthesized as a 57 kDa precursor (Bouvier *et al.*, 1994). Therefore, in transfected plants, the transit peptide was recognized and correctly processed, targeting the recombinant enzyme to the chloroplast. Under these conditions, chloroplast epoxy xanthophylls were efficiently converted into the non-chloroplast carotenoid capsanthin according to the pathway depicted in Figure 1.

Amino acid sequence comparison between Ccs and lycopene β-cyclase indicates that they have 60% identity (Hugueney *et al.*, 1995). Since the two plant enzymes have regions of homology, we decided to investigate whether virus-derived antisense *Ccs* RNA could cause chloroplast accumulation of lycopene, a chromoplast-specific carotenoid. Ten days after inoculation, the leaves from plants transfected with antisense *Ccs*$^{-}$ developed wild-type TMV symptoms and did not accumulate detectable amounts of lycopene.

At the cellular level, we analysed the effects of *Ccs* expression on the structure of chloroplast membranes. Electron micrographs of tissue sections from non-infected (control) and leaves transfected with antisense *Ccs*$^{-}$ revealed the presence of normal chloroplasts containing a well-developed array of grana (Figure 5). On the other hand, chloroplasts of leaves transfected with sense *Ccs*$^{+}$ displayed reduced grana stacks, pronounced vesicle formation, and distortion of thylakoid membranes. Similar structures can be seen during the early phase of chloroplast to chromoplast differentiation (Camara and Brangeon, 1981).

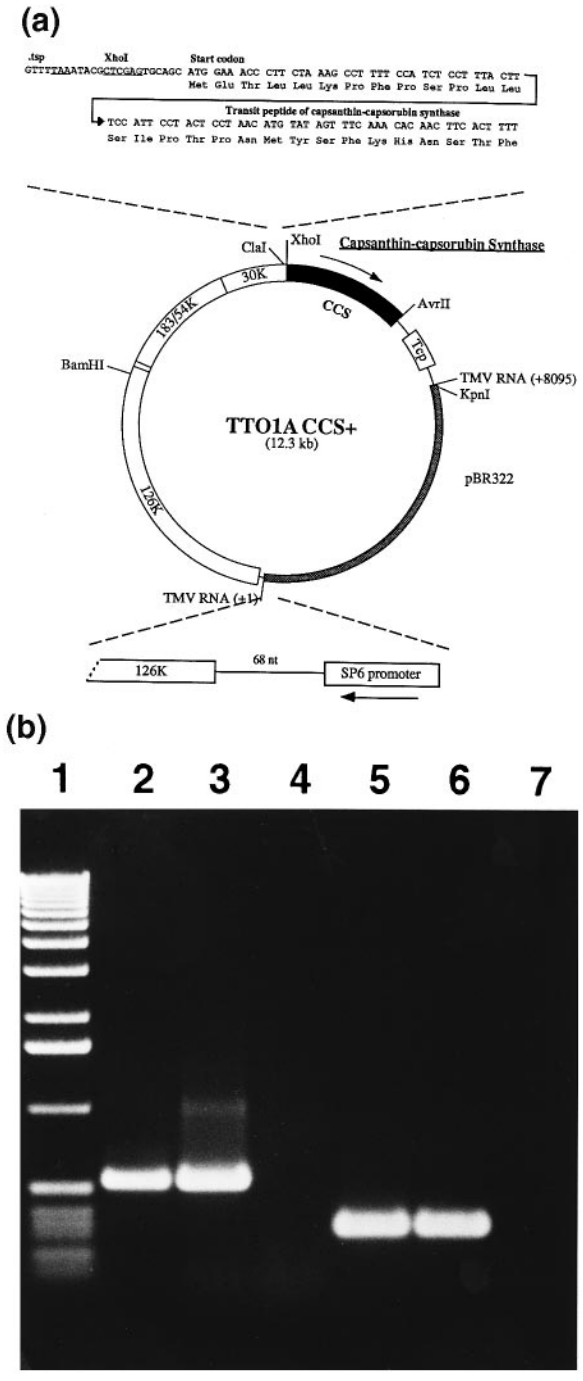

Figure 2. Capsanthin–capsorubin expression vector (TTO1A*Ccs*) and amplification of *Ccs* transcripts.
The plasmid contains the TMV-U1 open reading frame (ORF) encoding 126, 183/54 and 30 kDa, the ToMV coat protein gene (*Tcp*), the SP6 promoter, the pepper capsanthin–capsorubin synthase gene (*Ccs*), and part of the pBR322 plasmid. The TMV-U1 promoter located within the minus strand of the 30 kDa ORF directs the expression of *Ccs* in the sense (*Ccs*$^{+}$) (a) or antisense (*Ccs*$^{-}$) orientation (not shown).
(b) Amplification of Ccs from plants transfected with *in vitro* transcripts of TTO1A*Ccs*$^{+}$ and TTO1A*Ccs*$^{-}$. Lane 1 contains a 1 kb ladder (from 75 to 12 000 bp, Gibco BRL); lane 2; RT–PCR amplification products from systemically infected leaf transfected with TTO1A*Ccs*$^{+}$ (sense orientation); lane 3, plasmid TTO1A*Ccs*$^{+}$ PCR control; lane 4, uninoculated *N. benthamiana* amplified with *Ccs*$^{+}$ PCR primers; lane 5; RT–PCR amplification products from systemically infected leaf transfected with TTO1A*Ccs*$^{-}$ (antisense orientation); lane 6, plasmid TTO1A*Ccs*$^{-}$ PCR control; lane 7, uninoculated *N. benthamiana* amplified with *Ccs*$^{-}$ PCR primers.

Functional integration of capsanthin into photosynthetic membranes

In higher plant chloroplasts, the bulk of xanthophylls is associated with LHCII in relatively constant stoichiometric

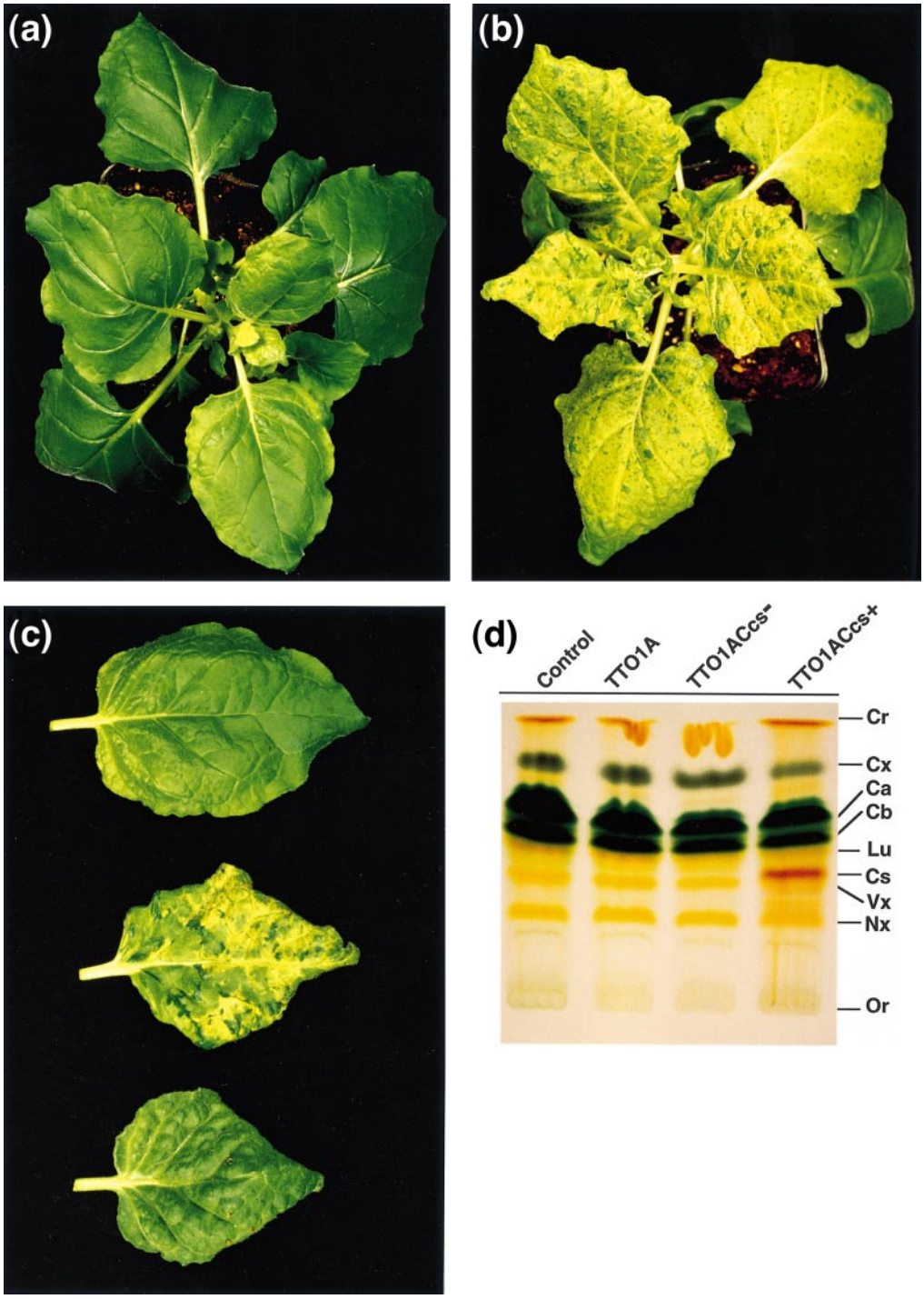

Figure 3. Phenotype and pigment changes following expression of capsanthin–capsorubin synthase (Ccs) in transfected *N. benthamiana* using TTO1A*Ccs*. (a) Control plants; (b) transfected plant (using TTO1A*Ccs*$^{+}$); (c) from top to bottom: control leaf, transfected leaf (using TTO1A*Ccs*$^{+}$), transfected leaf (using TTO1A*Ccs*$^{-}$); (d) chromatographic analysis of chloroplast pigments in transfected *N. benthamiana* using TTO1A*Ccs*. Total pigment extract (2 mg) was separated by thin-layer chromatography on a silica gel plate using hexane/acetone (60/40, v/v) as developing solvent. From left to right: pigment extract from uninoculated plants and from transfected plants using empty vector, TTO1A*Ccs*$^{-}$ and TTO1A*Ccs*$^{+}$. Cr, carotenes; Cx, chlorophyll degradation products; Ca, chlorophyll *a*; Cb, chlorophyll *b*; Lu, lutein; Cs, capsanthin; Vx, violaxanthin; Nx, neoxanthin; Or, the origin.

amounts with chlorophylls and apoproteins (Thornber *et al.*, 1987). In our study, the heterologous expression of *Ccs* in *N. benthamiana* caused a dramatic change in the carotenogenic pathway. Since approximately 36% of the total carotenoids were converted to capsanthin, we decided to investigate whether capsanthin is sequestered, integ-

Table 1. Carotenoid and chlorophyll contents of control and transfected *N. benthamiana*

Transfected plants	Carotenoid composition[1]							Total pigments[b]	
	β-carotene	Lutein	Antheraxanthin	Capsanthin	Capsorubin	Violaxanthin	Neoxanthin	Carotenoids	Chlorophylls
Control	14	40	4	0	0	20	22	184 ± 12	1640 ± 85
Empty vector	12	41	4	0	0	20	23	184 ± 8	1304 ± 67
TT01A*Ccs*$^-$	14	44	5	0	0	16	21	144 ± 10	1360 ± 52
TT01A*Ccs*$^+$	17	20	0	36	2	8	17	168 ± 14	1256 ± 54

[a]Carotenoid composition expressed as percentage of total carotenoids. The value given is based on the mean of five total pigment determinations.
[b]Total pigments (carotenoid and chlorophyll) content expressed as μg per g fresh weight. The value given is the mean of five experiments.

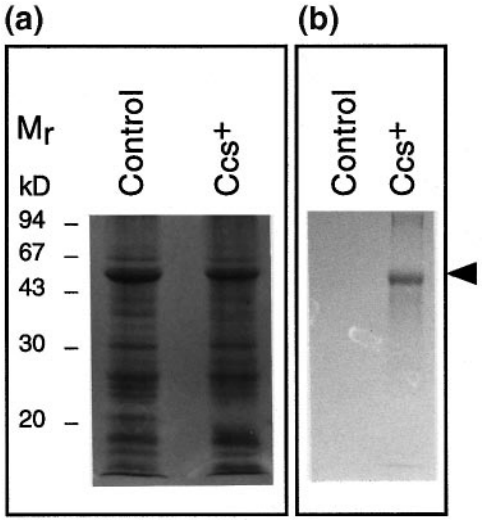

Figure 4. SDS–PAGE and Western blot analysis of Ccs from transfected *N. benthamiana* using TTO1A*Ccs*$^+$.
(a) SDS–PAGE analysis and (b) Western blot analysis of total plastid proteins. From left to right: uninoculated and transfected (TTO1A*Ccs*$^{+)}$ plants.

rated into thylakoid membranes, or specifically recruited into photosynthetic pigment–protein complexes.

A general prerequisite to sequestration of excess xanthophylls is the esterification of free hydroxyl groups. Data reported above (Figure 3d) are consistent with the absence of esterification of capsanthin. Furthermore, the electron microscope image (Figure 5) did not reveal the presence of fibrils, globules or crystals, which represent the major sink for sequestration excess carotenoids in plastids (Camara *et al.*, 1995). Based on this evidence, we analysed the integration of capsanthin into thylakoid membrane pigment–protein complexes. Solubilized pigment–protein complexes were resolved into four main bands corresponding to photosystem I holocomplex (PSI), core complex (CCII), trimeric (LHCII)3 and monomeric (LHCII) light-harvesting complex (Figure 6), according to a previously described procedure (Dreyfuss and Thornber, 1994). The band corresponding to free pigment was negligible in control and transfected leaves (Figure 6). Collectively, two conclusions can be drawn from these data. First, non-native capsanthin did not appreciably change the migration profile of the thylakoid pigment–protein complexes. Second, capsanthin is integrated into pigment–protein complexes. To determine the location of capsanthin within the pigment–protein complexes, the different green bands were excised, their pigment contents were eluted, and then analysed by HPLC. The pigment composition of the different photosynthetic complexes is shown in Table 2. Capsanthin is exclusively incorporated into (LHCII)3 and LHCII, i.e. in complexes belonging to PSII antenna. This phenomenon is specific and has been reproduced in more than five different analyses.

The absorption and fluorescence excitation spectra of the pooled LHCs [(LHCII)3 and LHCII)] fractions from control and transfected plants with identical chlorophyll content are shown (Figure 7a,b). In both cases, the absorption spectrum parallels the fluorescence excitation spectrum, suggesting an efficient energy transfer. The *in vivo* contributions of chlorophyll *b* and xanthophyll are generally estimated from the fluorescence excitation at 470 nm and 487 m (Siefermann-Harms and Ninnemann, 1982). Thus, the increased excitation between 470 and 487 nm in *Ccs*-transfected leaves could be attributed to an efficient energy transfer in the following order, *Ccs* > Chl*b* > Chl*a* (Figure 7b). This trend is reinforced by the fact that pretreatment of the LHC preparation of control and transfected plants with Triton X-100 abolishes the heights of the excitation spectrum (Figure 7a,b), in good agreement with previous data (Siefermann-Harms and Ninnemann, 1982).

Further analysis revealed that, on a chlorophyll basis, control and transfected leaves possess identical concentrations of PSI and PSII reaction centres (Table 3). The slight reduction observed on leaf area basis most probably reflects the reduction of the chlorophyll content in transfected leaves.

Discussion

Autoregulation of thylakoid carotenoid composition

We observed that in *N. benthamiana*, channelling the chloroplast carotenogenic pathway to synthesis of the non-

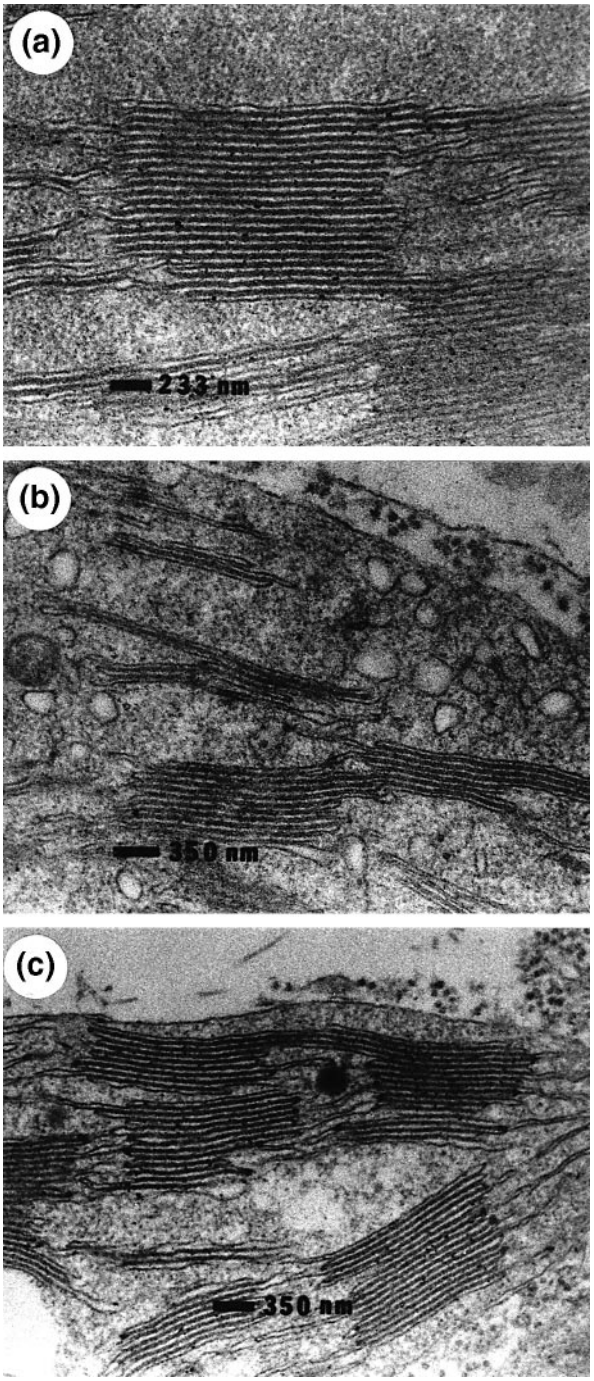

Figure 5. Electron micrographs of chloroplasts from control and TTO1A*Ccs*-transfected *N. benthamiana*.
(a) Uninoculated *N. benthamiana*.; (b) *N. benthamiana* transfected with TTO1A*Ccs*$^{+}$; (c) *N. benthamiana* transfected with TTO1A*Ccs*$^{-}$.

native carotenoid capsanthin changes the composition of the endogenous carotenoids. The reduction of the native xanthophylls lutein, antheraxanthin, violaxanthin and neoxanthin in *Ccs*$^{+}$-transfected leaves is stoichiometrically compensated by nearly identical amounts of capsanthin (Table 1). The decrease in lutein content could be caused

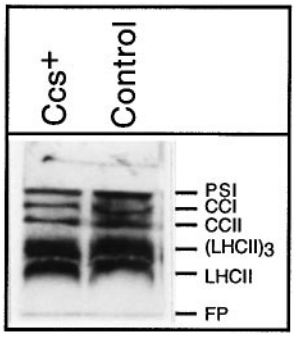

Figure 6. Pigment–protein complexes of control and TTO1A*Ccs*-transfected *N. benthamiana*.
Solubilized protein complexes equivalent to 20 μg of total chlorophylls were separated by non-denaturating deriphat gel elecrophoresis. PSI, photosystem I; CCI, core complex I; CCII, core complex II; (LHCII)3, trimeric light-harvesting complex II; LHCII, monomeric LHCII; FP, free pigment.

by the increased β-carotene synthesis from lycopene (Figure 1) by Ccs which has both β and κ cyclase activities (Bouvier *et al.*, 1997; Hugueney *et al.*, 1995). One can hypothesize that an auto-adjustment mechanism operates in chloroplasts, in contrast to chromoplasts, which probably regulates the pigment to protein ratio at levels compatible with photosynthetic activity. Indeed, a similar phenomenon has been observed in tobacco (Marin *et al.*, 1996) and in *Arabidopsis* epoxy xanthophyll-deficient mutants (Rock and Zeevart, 1991; Rock *et al.*, 1992) which are photosynthetically competent. In both mutants, the depletion of violaxanthin and neoxanthin was compensated for by an increase of zeaxanthin content. Further evidence for this autoregulation process is observed in *Arabidopsis* lutein-deficient mutants which accumulate increased amounts of zeaxanthin, antheraxanthin and violaxanthin (Pogson *et al.*, 1996). The enhanced formation of capsanthin indicates that a significant proportion of chloroplast epoxy-xanthophylls are relatively accessible, in good agreement with previous data based on the analysis of pigment–protein complexes (Brown, 1987; Peter and Thornber, 1991).

The overall phenotype of leaves accumulating capsanthin is reminiscent of the tomato mutant *green flesh* (Cheung *et al.*, 1993) or the pepper mutants cultivar sweet chocolate (Deruère *et al.*, 1994) or mulato (unpublished results) in which the chloroplasts differentiate into chlorochromoplasts in which the thylakoid system is well preserved. In these mutants, we observed that, following mild treatment of purified plastids with 0.5–1% dodecylmaltoside followed by centrifugation at 15 000 ***g*** for 10 min, the bulk of capsanthin ester and capsorubin ester were associated with the pellet while the total chlorophylls and the unesterified xanthophylls were recovered in the supernatant fraction. These data suggest the existence of two spatially distinct carotenogenic pathways in the same chlorochromoplast. These results are corroborated by the fact that, in these chlorochromoplasts, a set of normal

Table 2. Carotenoid and chlorophyll compositions of pigment–protein complexes of control and transfected *N. benthamiana*

Pigment–protein complex	Transfected plants	Carotenoids[a]						Chlorophylls[a]	
		β-carotene	Lutein	Antheraxanthin	Capsanthin	Violaxanthin	Neoxanthin	Chl*a*	Chl*b*
PSI	Control	19.7 ± 1.7	6.8 ± 0.07	0.4 ± 0.01	0	1.4 ± 0.08	0.7 ± 0.04	58.0 ± 2.3	13.0 ± 0.8
	TT01A*Ccs*$^+$	19.8 ± 2.1	6.4 ± 0.08	0	0	1.4 ± 0.07	0.4 ± 0.03	59.0 ± 2.4	13.0 ± 0.5
CCI	Control	18.0 ± 1.4	5.0 ± 0.04	0	0	1.6 ± 0.09	0.4 ± 0.02	60.0 ± 2.4	15.0 ± 0.9
	TT01A*Ccs*$^+$	17.2 ± 1.3	5.2 ± 0.06	0	0	1.9 ± 0.05	0.6 ± 0.01	62.1 ± 2.2	13.0 ± 0.6
CCII	Control	17.0 ± 1.1	4.0 ± 0.05	0	0	0.7 ± 0.04	0.5 ± 0.02	70.0 ± 3.6	7.8 ± 0.4
	TT01A*Ccs*$^+$	14.5 ± 1.0	4.0 ± 0.09	0	0	0.6 ± 0.07	0.6 ± 0.02	72.8 ± 2.5	7.5 ± 0.3
(LHCII)3	Control	0.2 ± 0.01	13.0 ± 0.08	0.3 ± 0.02	0	7.5 ± 0.06	4.0 ± 0.06	40.0 ± 2.3	35.0 ± 1.5
	TT01A*Ccs*$^+$	0.2 ± 0.02	7.7 ± 0.05	0	8.2 ± 0.04	3.0 ± 0.08	4.2 ± 0.05	39.0 ± 2.2	37.7 ± 1.8
LHCII	Control	0.2 ± 0.03	12.1 ± 0.03	0.2 ± 0.01	0	4.8 ± 0.05	3.7 ± 0.02	48.6 ± 2.1	30.4 ± 2.0
	TT01A*Ccs*$^+$	0.1 ± 0.04	6.0 ± 0.06	0	11.2 ± 0.08	1.4 ± 0.07	3.3 ± 0.02	48.6 ± 2.4	29.4 ± 1.7

[a]Carotenoid and chlorophyll composition are expressed as mol percentage of total pigments (carotenoids + chlorophylls) of each pigment–protein complex. The value given is the mean of five experiments.

Table 3. Reaction centres of control and transfected *N. benthamania*[a]

Plants	PSI		PSII	
	Chlorophyll per P700 (mol Chl P700 mol^{-1})	P700 per leaf area (μmol P700 m^{-2})	Chlorophyll per atrazine bound (mol Chl atrazine mol^{-1})	Atrazine bound per leaf area (μmol Chl atrazine m^{-2})
Control	350 ± 5	1.02 ± 0.02	539 ± 8	0.95 ± 0.03
TT01A*Ccs*$^+$	345 ± 9	0.72 ± 0.01	528 ± 7	0.81 ± 0.04

[a]Data show the mean ± SD of four different experiments.

thylakoid membranes co-exists with a distinct set of achlorophyllous structures. When *N. benthamiana* chloroplasts were subjected to osmotic shock in the presence of 50 mM Tris–HCl (pH 7.6), followed by centrifugation, no release of capsanthin in the supernatant was observed. On the other hand, when the dodecylmaltoside solubilization procedure was adopted, we noted that the bulk of xanthophylls including capsanthin and the total chlorophylls were in the supernatant. These data suggest two conclusions. First, in leaves transfected with *Ccs*$^+$, the chloroplasts could not be assimilated to true chromoplasts. Second, in *Ccs*$^+$-transfected leaves, capsanthin is probably integrated into the thylakoid membranes like normal ε and β chloroplast carotenoids. The absence of an esterified hydroxyl group in capsanthin reinforces this phenomenon, since incorporation of carotenoids into photosynthetic membranes is aided by the presence of polar groups (Chadwick and Frank, 1986).

Preferential integration of non-native carotenoids into photosystem II complexes

Our data indicate that chloroplast thylakoids can accommodate non-native capsanthin and this phenomenon is paralleled by a characteristic distortion of the grana stacks. Could these changes be induced by capsanthin? In the absence of detailed studies, it is pertinent to note that in bacteria and algae the diversity of photosynthetic membrane architecture is associated with the integration of structurally different carotenoid molecules (Siefermann-Harms, 1985). Therefore, the simplest interpretation of our results is that steric hindrance provoked by capsanthin changes the normal architecture of the thylakoid membranes. This conclusion is consistent with the severe disorganization of thylakoid membranes in mutant maize accumulating lycopene (Faludi-Daniel *et al.*, 1968) or ζ-carotene (Faludi-Daniel, 1975) and is reinforced by the fact that carotenoid structures affect photosynthetic membrane shaping in prokaryotes (Lang and Hunter, 1994). Pigment–protein analysis revealed that capsanthin is essentially integrated into PSII complexes rather than PSI. This propensity is reminiscent of the fact that the carotenoid requirement is much more stringent for PSII than for PSI (Markgraf and Oelmüller, 1991).

It has been shown that foreign carotenoids incorporated in bacterial reaction centre complexes or in antenna complexes (Hunter *et al.*, 1994) adopt a conformation compatible with energy transfer to bacteriochlorophylls. The concomitant autoregulation of the total carotenoid synthesis, the specific recruitment of capsanthin into LHCII and its contribution to the fluorescence excitation spectrum of LHCII in transfected plants suggest that a similar process

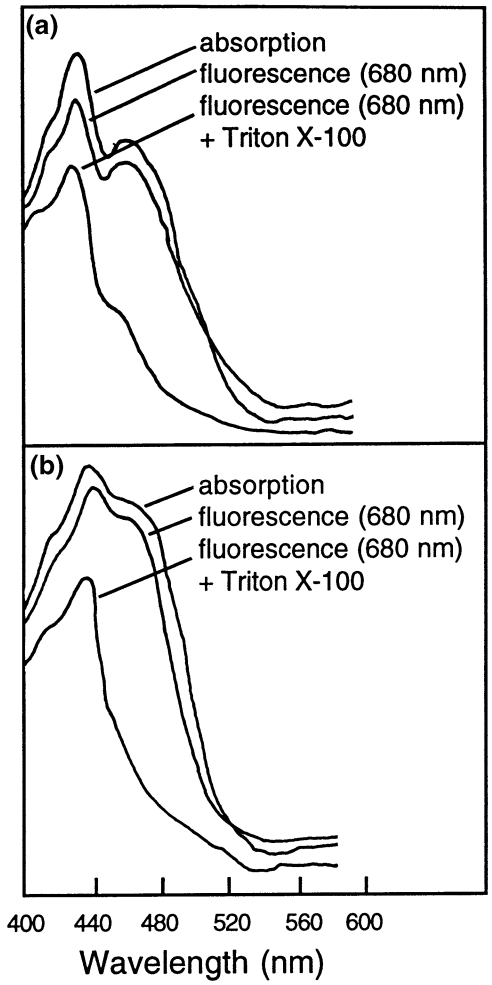

Figure 7. Absorbance and fluorescence excitation spectra of LHC [(LHC)3 + LHC] from control and TTO1A*Ccs*$^+$-transfected *N. benthamiana*. Trimeric and monomeric LHC were isolated as described in Experimental procedures and adjusted to 7.5 μg of chlorophyll before recording the absorption and the fluorescence excitation spectra (680 nm emission) in 50 mM Tris–HCl buffer (pH 7.6) containing 100 μg of digitonin per μg of chlorophyll. Where shown, Triton X-100 was used at a concentration of 0.5% to alter the energy transfer between the pigments. (a) Uninoculated *N. benthamiana*.; (b) *N. benthamiana* transfected with TTO1A*Ccs*$^+$.

may operate in plant thylakoid membranes. This conclusion is reinforced by the fact that, on a chlorophyll basis, the concentrations of PSI and PSII reaction centres were identical in control and transfected *N. benthamiana*. However, our data do not exclude the possibility that the reduced lutein content still plays a photochemical role. The recruitment of massive amount of capsanthin into LHCII is suggestive of the high selective pressure for the differentiation of photosynthetic structures in chloroplasts and of presumptive evidence that during evolution of phototrophs, divergent modification of photosynthetic membrane constituents was less stringent for the pigment composition of antenna complexes in contrast to core complexes. This is reflected by the fact that in the red alga *Porphyridium cruentum*, phycobilisomes co-exist with antenna complexes immunologically related to plant LHC and fucoxanthin–chlorophyll protein complexes (Wolfe *et al.*, 1994). The recruitment of carotenoids in these photochemical complexes could be facilitated by two facts. First, the binding sites of carotenoids probably display broad specificity (Hurry *et al.*, 1997; Moskalenko *et al.*, 1991). Second, it has been shown that, except for lutein, chloroplast xanthophylls are probably located at the periphery of LHCII complexes (Kühlbrandt *et al.*, 1994).

Finally, introduction of non-native antenna pigments in plant chloroplasts has not been explored previously. The intense *de novo* synthesis and functional incorporation of capsanthin in transfected leaves demonstrates that the viral-based expression system can be used as a powerful tool to quickly and deliberately re-route the carotenogenic pathway in plants. Thus, in a wider context, rational design of individual pigment components offers a possibility for manipulating the architecture and the photosynthetic competence of thylakoid membranes. To this end, carotenoids with extended chromophores could broaden the absorption spectra of antenna complexes to regions of the visible light poorly explored by higher plants in contrast to alga, and also provide a better photodynamic screen against photo-oxidation (Bidigare *et al.*, 1993; Hagen *et al.*, 1994). Indeed, some plants belonging mostly to gymnosperms (Ida, 1981) actively synthesize the keto-carotenoid rhodoxanthin in response to photo-oxidative stress conditions. The strategy used promises to be a valuable tool for further predictable manipulations of plastid functions.

Experimental procedures

Plasmid constructions

A cDNA encoding bell pepper Ccs was modified by PCR using the following oligonucleotides: 5′- GCCTCGAGTGCAGCATGGAAACCCTTCTAAAGCCTTTCC-3′ (upstream), and 5′-TCCCTAGGTCAAAGGCTCTCTATTGCTAGATTGCC-3′ (downstream). The 1.6 kb *Xho*I–*Avr*II cDNA fragment was placed under the control of the TMV-U1 coat protein subgenomic promoter by subcloning into TTO1A, creating plasmid TTO1A*Ccs*$^+$ (sense orientation). A partial cDNA encoding bell pepper Ccs (Pro279–Val367) was modified by PCR using the following oligonucleotides: 5′-CGCCTAGGCCAACATTCTTGTATGCAATGCC-3′ (upstream), and 5′- CGCTCGAGCACCATGTACCCAGACGATGGATGAAC-3′ (downstream). The partial bell pepper *Ccs* cDNA was subcloned into TTO1A at the *Xho*I–*Avr*II sites, creating plasmid TTO1A*Ccs*$^-$ (antisense orientation).

In vitro *transcriptions, inoculations, and analysis of transfected plants*

N. benthamiana plants were inoculated with *in vitro* transcripts as described previously (Kumagai *et al.*, 1995). RNA was isolated from transfected plants and amplified by RT–PCR using conditions

described previously, except that the annealing temperature was 50°C instead of 55°C (Kumagai *et al.*, 1995). The following oligonucleotides were used in the RT–PCR reactions: TMV-U1, 5′-TAATCGATGATGATTCGGAGGCTAC-3′ (upstream primer for TTO1A*Ccs*$^+$, TTO1A*Ccs*$^-$, and uninoculated *N. benthamiana* control); *Ccs*V164, 5′-TACTCTACCATATGGTCTGTCC-3′ (downstream primer for TTO1A*Ccs*$^+$, uninoculated *N. benthamiana Ccs*$^+$ control); *Ccs*P279A, 5′-CGCCTAGGCCAACATTCTTGTATGCAATGCC-3′ (downstream primer for TTO1A*Ccs*$^-$, uninoculated *N. benthamiana Ccs*$^-$ control).

Immunological detection of Ccs from transfected N. benthamiana

Twelve days after inoculation, total chloroplast proteins were subjected to SDS–PAGE analysis followed by Western blotting using Ccs (Bouvier *et al.*, 1994) antibodies as described previously (Camara, 1993).

Pigment analysis

Twelve days after inoculation, upper leaves from 12 plants were harvested and lyophilized. The total lipids were extracted (Folch *et al.*, 1957) and the resulting non-saponified extract was evaporated to dryness under argon, weighed (to determine the total lipid content), and analysed for pigment composition by HPLC (Bouvier *et al.*, 1994). Alternatively, as specified in the text, the pigments were separated by thin-layer chromatography on silica gel G using hexane/acetone (60/40 v/v). Further identification criteria were as described previously (Camara, 1980; Davies, 1976).

Pigment–proteins and reaction centre analysis

Pigment–proteins were isolated as described previously (Dreyfuss and Thornber, 1994) using thylakoid membranes obtained from osmotically disrupted plastids (Camara, 1993). Following electrophoresis, each pigment–protein band was eluted with dimethylformamide and the pigment content transferred to diethyl ether before HPLC analysis as described above. Alternatively, the green bands corresponding to LHCs were electro-eluted using an Isco apparatus and the resulting eluate was equilibrated by dialysis in 50 mM Tris–HCl buffer (pH 7.6) supplemented with digitonin (100 μg per μg of chlorophyll) as described previously (Siefermann-Harms and Ninnemann, 1982). The absorption spectra were recorded with a Hitachi 356 spectrophotometer and the fluorescence spectra were monitored with a Hitachi MPF-4 fluorescence spectrophotometer. The isolated thylakoids were used to quantify the PSI and PSII reaction centres. The concentration of PSI was estimated from the content of photo-oxidizable P700 (Hiyama and Ke, 1972). The concentration of PSII reaction centres was evaluated using the number of atrazine binding sites (Tischer and Strotmann, 1977) using [$U^{14}C$]-atrazine (10 mCi mmol^{-1}, Sigma). The chlorophyll concentration was determined as described previously (Arnon, 1949).

Electron microscopy

Leaf tissue was embedded in Spurr's low-viscosity epoxy embedding medium after fixation in 3% glutaraldehyde (buffered with 0.01 M Na-K phosphate buffer, pH 7.2) and post-fixation with 2% OsO_4 for 1 h. The embedded tissue was then sectioned, stained for 20 min in uranyl acetate (30% w/v in absolute methanol), and examined with a Zeiss CEM 902 transmission electron microscope at 80†kV.

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