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Identification of a novel gene coding for neoxanthin synthase from *Solanum tuberosum*

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Abstract The polymerase chain reaction analysis of potato plants, transformed with capsanthin capsorubin synthase *ccs*, revealed the presence of a highly related gene. The cloned cDNA showed at the protein level 89.6% identity to CCS. This suggested that the novel enzyme catalyzes a mechanistically similar reaction. Such a reaction is represented by neoxanthin synthase (NXS), forming the xanthophyll neoxanthin, a direct substrate for abscisic acid formation. The function of the novel enzyme could be proven by transient expression in plant protoplasts and high performance liquid chromatography analysis. The cloned NXS was imported in vitro into plastids, the compartment of carotenoid biosynthesis. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Neoxanthin synthase; Abscisic acid; Carotenoid; Violaxanthin; Capsanthin capsorubin synthase

1. Introduction

Significant process has been made during recent years in the molecular identification of genes involved in carotenoid biosynthesis in both plants and micro-organisms. In plants, almost all of the 'classical' structural genes are known to date and their sequences are available in the databases, including isopentenyl diphosphate/dimethylallyl diphosphate synthase isomerase, geranylgeranyl diphosphate synthase, phytoene synthase, phytoene desaturase, ζ-carotene desaturase, lycopene α- and β-cyclase, β-carotene hydroxylase and the subsequent epoxidase. One of the few yet unidentified genes is the one coding for neoxanthin synthase (NXS). The corresponding enzyme catalyzes the conversion of the di-epoxidated precursor violaxanthin into a xanthophyll carrying an allenic double bond, named neoxanthin which represents the classical final step in plant xanthophyll formation (Fig. 1).

To understand neoxanthin biosynthesis is of larger general interest, since this xanthophyll may represent in vivo the ultimate precursor in the formation of the plant hormone abscisic acid (ABA): there is a large and significant body of evidence favoring the so-called 'indirect' route towards ABA formation, namely via xanthophylls. It has been shown that the *vp14* gene product represents a dioxygenase catalyzing the cleavage of 9-*cis*-epoxy carotenoids to form C25 apo-aldehydes and xanthoxin [1]. Both violaxanthin and neoxanthin were accepted as substrates as well as 9-*cis* zeaxanthin (the latter not yielding xanthoxin) so that the *cis* configured double bond appeared to be the determinant for cleavage. The question arises, which of the two epoxidated xanthophylls plays the role as a precursor in vivo.

The identification of NXS has several hindrances. First, this reaction is not readily accessible by classical methods of protein purification, since it has hitherto not been possible to perform the reaction in vitro starting from exogenous violaxanthin. Moreover, it is to be expected to occur membrane bound, and dependent on membrane structures for enzymatic activity, as suggested by the high degree of lipophilicity of its substrate and product. Other methods like color complementation in *Escherichia coli* that have proven to be successful with other carotenoid biosynthetic enzymes are ruled out, because of the very high spectral similarity of violaxanthin and neoxanthin and the incapability of transformed *E. coli* to synthesize epoxidated xanthophylls in vivo [2]. Therefore, the identification of a mutant defective in this biosynthetic step was expected to lead to an identification. Alternatively, this could be accomplished as a matter of serendipity, and we report here its cloning and identification from an approach planned to answer a completely different question.

2. Materials and methods

2.1. Primers

Upstream I: 5′-GACGTGATCATCATTGGA-3′ (codes for: DVIIIG); downstream I: 5′-ATGGCTCATTACCTAAAT-3′ (codes for: HLGNEP); downstream II: 5′-TTCAAAGGCTCTCTATTGC-3′ (codes for: AIESL*); Neox 5′: 5′-ATGAGACGCACAAACTAATAT-3′ (codes for: METLLKPL); Neox 3′: 5′-ACCCCATGAGCAATTTGATA-3′ (codes for: YQIAHGV); up: 5′-TATCTGCAGAGCTCGCCCTT-3′; down: 5′-CCCTCGAGGATCCACGGTAT-3′.

2.2. Cloning procedures

Genomic DNA was isolated from leaves using the DNAeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Polymerase chain reaction (PCR) was carried out for 32 cycles (1 min at 94°C, 1 min at 52°C and 40 s at 72°C) using 200 ng DNA and the primers upstream I and downstream I. The PCR product (562 bp) from control plants was purified using the GFX™ PCR DNA and Gel Band Purification Kit (Pharmacia; Freiburg,

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Abbreviations: ABA, abscisic acid; CCS, capsanthin capsorubin synthase; LCY, lycopene cyclase; MeOH, methanol; NXS, neoxanthin synthase

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Germany) according to the instructions of the manufacturer. The PCR product was then blunt-ended using T4 DNA polymerase (Boehringer; Mannheim, Germany) according to [3], ligated into *Eco*RV digested pBluescriptKS and sequenced.

Total RNA from leaves was isolated using the Plant RNeasy Kit (Qiagen; Hilden, Germany) according to the manufacturer's protocol and the *nxs* was cloned using reverse transcription (RT)-PCR in two steps. First, a fragment of 1252 bp, coding for the amino acids 83–498 was amplified. In the second step the 5′ end was amplified and ligated to the 3′ end. RT was carried out according to the instructions of the manufacturer with 500 ng total RNA in the presence of 100 ng downstream II primer using SuperScript II (Gibco-BRL, Eggenstein, Germany) in a total volume of 20 μl. For amplification, 10 μl of the RT assay were added to a PCR master mix containing: 100 ng upstream I, 50 ng downstream II, the delivered buffer and 1 U *Taq* DNA polymerase (Pharmacia; Freiburg, Germany). PCR was carried out for 32 cycles (1 min at 94°C, 1 min at 52°C, 1.5 min at 72°C) and the PCR product was cloned as described above, to give pNix10. The 5′ end was then amplified using RT-PCR as described above and the primers Neox 5′ and Neox 3′. The fragment obtained was ligated into pBAD-TOPO (Invitrogen; Groningen, The Netherlands) according to the instructions of the manufacturer yielding pNeo5′, and sequenced. To obtain the full length cDNA, a 481 bp DNA fragment carrying the 5′ end was excized from pNeo5′ using the *Nde*I and *Xba*I sites and ligated into pNix10 yielding pNEOXBlue.

2.3. Transient expression of NXS in plant protoplasts

A DNA fragment carrying the full length cDNA was amplified from pNEOXBlue using the primers Up and Down to introduce *Sac*I and *Bam*HI sites in the multicloning site. The PCR was carried out using 20 ng DNA, 100 ng of each primer, 1 U proof-reading *Pwo* polymerase (PeqLab, Erlangen, Germany) in the delivered buffer and in the presence of 2 mM dNTPs for 30 cycles (1 min at 94°C, 1 min at 50°C and 90 s at 68°C). The PCR product was purified as above and then digested with *Sac*I and *Bam*HI and ligated into pRT99 (unpublished) under the control of the constitutive 35S CaMV promoter yielding pRTNix99.

The isolation of mesophyll protoplasts from *Nicotiana tabacum* cv. Petit Havana SR_1 and the polyethylene glycol (PEG)-mediated transformation with pRTNix99 were performed according to the protocol of Spangenberg and Potrykus [5] with the modification that each assay contained 1.5×10^6 protoplasts that were treated with 60 μl DNA (1 μg/μl) and 250 μl PEG. The control cells used in carotenoid analysis were transformed with the plasmid pGN35Sluc [6] under otherwise identical conditions. Transient expression analysis of *nxs* was also performed in protoplasts derived from an *Arabidopsis thaliana* cell culture after electroporation [7] with 10 μg/10^6 cells effector plasmid pRTNix99 and 5 μg 35S/LUC. Transformation efficiency was monitored in vivo by measuring luciferase activity. With *A. thaliana*, transformed protoplasts were subjected to high performance liquid chromatography (HPLC) analysis 4, 6, 8 and 12 h after transformation. Transformed tobacco protoplasts were analyzed 6 h after transformation.

2.4. Carotenoid analysis

Protoplasts were extracted with $CHCl_3$/methanol (MeOH) (2:1, v/v) to complete decolorization. The extracts were dried under a stream of nitrogen and dissolved in 50 μl of $CHCl_3$. A 10 μl aliquot was then

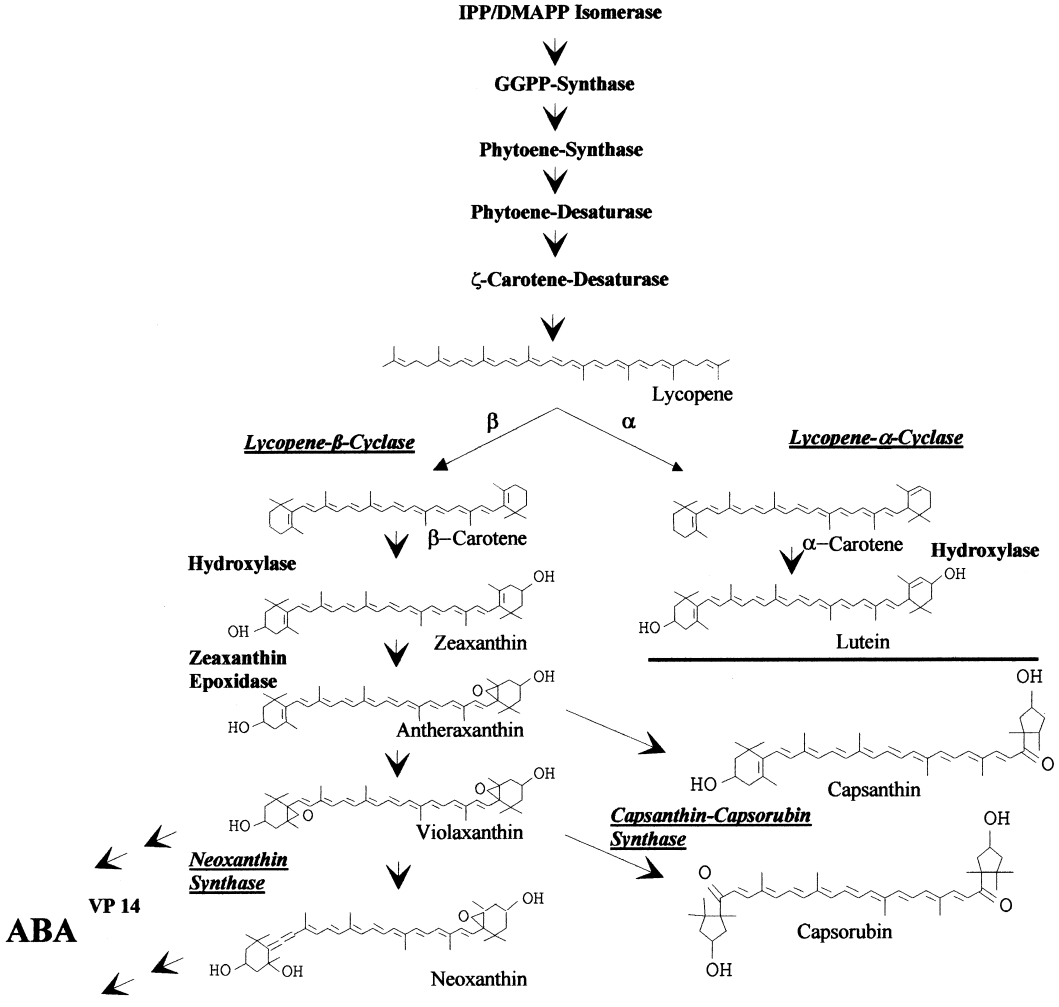

Fig. 1. Biosynthetic pathway leading to the formation of neoxanthin, capsanthin and ABA. Homologous enzymes are given in italic and are underlined; for further explanations see text.

subjected to HPLC analysis using a C_{30}-reversed phase HPLC column (YMC Europe) with the solvent system A: MeOH/water (16:1, v/v) and B: *tert*-butylmethyl ether/MeOH (1:1, v/v). The column was developed at a flow-rate of 1 ml/min with 100% A isocratically for 25 min, followed by a linear gradient to 100% B for 50 min. Alternatively, separations were carried out on an adsorptive phase using a Hypersil-Si-column (Knaur, Germany) developed isocratically with hexane/diethylether/ethanol (400:100:15, v/v/v) as the eluent at a flow-rate of 1.3 ml/min. Carotenoids were identified by using authentic references isolated from daffodil flowers and spinach leaves and by their absorption spectra as recorded by a photodiode array detector (Waters, USA). Since the spectral difference between neoxanthin and violaxanthin is very small and several isomers were resolved, the identity of neoxanthin was proven by carrying out acid catalyzed epoxide–furanoid rearrangement reactions. Collected individual peaks and standards were transferred into chloroform and a catalytic amount of diluted HCl was added. This resulted in an about 20 nm hypochromic shift for neoxanthin while the shift was about 40 nm with violaxanthin, as expected.

2.5. In vitro transcription, translation and plastid-import

The full length cDNA was excized from pNEOXBlue with *Kpn*I and *Xba*I and ligated into pGEM3 (Serva, Heidelberg, Germany) under the control of the SP6 promoter yielding pNIXGEM3. In vitro transcription, translation and protein import were carried out as described previously [4].

3. Results

3.1. Potatoes contain a novel, capsanthin capsorubin synthase (ccs) homologous gene

In *Capsicum annuum* fruit, CCS is strongly expressed, in contrast to all other carotenoid biosynthetic enzymes examined so far [8]. We hypothesized that the high abundance of this final enzyme in the multi-step carotenogenic pathway would lead to an accelerated flux of metabolites through the pathway by creating chemical dizequilibria. Therefore, a cDNA clone coding for CCS (kindly provided by B. Camara, Strasbourg, France) was cloned into pBin 33 (kindly provided by C. Gatz, Göttingen, Germany), under the control of the tuber-specific patatin B33 promoter [9]. *Agrobacterium* mediated transformation of potato (cv. Desirée) leaf-discs was performed and regenerated plantlets were selected for kanamycin resistance. The transgeneity of the obtained plants was checked by PCR. Using primer pair I and genomic DNA, we were able to amplify a fragment of the expected size in transgenic plants. However, this was also found when DNA from untransformed controls was used. Sequencing of the fragment obtained from control plants revealed a large degree of homology with *ccs*. Since neither capsanthin nor capsorubin are present in potato, we suspected that we had a related, but novel gene in our hands. By using RT-PCR (see Section 2) we obtained the corresponding cDNA clone. The sequencing of the cDNA revealed a novel sequence (accession number AJ272136) that shared at the level of the amino acid sequence an identity of 89.6% with the sequence from CCS and 64% identity with β-lycopene cyclase (β-LCY) (both from *C. annuum*; Fig. 2). It appeared thus probable that the novel cDNA coded for an enzyme that carries out a reaction or utilizes a reaction mechanism that shares a high degree of similarity with the one present in LCY or CCS. Besides lycopene cyclization, the most similar carotenoid metabolizing reaction which is common in potato is represented by the synthesis of neoxanthin from violaxanthin (Fig. 3, see also Section 4). We therefore assumed the novel sequence to code for NXS.

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NXS    METLLKPLTSLLLSSPTPHRSIFQQN-----PPSLNPTTK    35
CCS    METLLKPFPSPLLSIPTPNMYSFKHN-----STFPNPTKQ    35
CAPS   MDTLLR----------TPNNLEFLHGFGVKVSAFSSVKSQ    30
       *.***.          **.   * ..     ..  . ...

NXS    KKSRKCHFRNESSKLFC------SFLDLAPISKPESFDVN    69
CCS    KDSRKFHYRNKSSTHFC------SFLDLAPTSKPESLDVN    69
CAPS   KFGAKKFCEGLGSRSVCVKASSSALLELVPETKKENLDFE    70
       * . *    . .*   *      ..*.*.* .* *..* .

NXS    ISLVDPNSGRAQFDVIIIGAGPAGLRLAEHVSKYGIKVCC   109
CCS    ISWVDTDLDGAEFDVIIIGTGPAGLRLAEQVSKYGIKVCC   109
CAPS   LPMYDPSKGVV-VDLAVVGGGPAGLAVAQQVSEAGLSVCS   109
       ..  *.. . .  *. ..*.***** .*..**. *..**.

NXS    VDPSPLSMWPNNYGVWVDEFENLGLEDCLDHKWPMTCVHI   149
CCS    VDPSPLSMWPNNYGVWVDEFEKLGLEDCLDHKWPVSCVHI   149
CAPS   IDPNPKLIWPNNYGVWVDEFEAMDLLDCLDATWSGAAVYI   149
       .**.*  .************* ..* **** .*. . *.*

NXS    NDHKTKYLGRPYGRVSRKKLKLRLLNSCVENRVKFYKAKV   189
CCS    SDHKTKYLDRPYGRVSRKKLKLKLLNSCVENRVKFYKAKV   189
CAPS   DDKTTKDLNRPYGRVNRKQLKSKMMQKCILNGVKFHQAKV   189
       .*..** *.******.**.** .....*. * ***..***

NXS    WKVEHEEFESSIVCDDGKKIRGSLVVDASGFASDFIEYDK   229
CCS    LKVKHEEFESSIVCDDGRKISGSLIVDASGYASDFIEYDK   229
CAPS   IKVIHEESKSMLICNDGITIQATVVLDATGFSRSLVQYDK   229
        ** *** .* ..*.** .* .....**.*.......***

NXS    PRNHGYQIAHGVLVEVDNHPFDLDKMVLMDWRDSHLGNEP   269
CCS    PRNHGYQVAHGILAEVDNHPFDLDKMMLMDWRDSHLGNEP   269
CAPS   PYNPGYQVAYGILAEVEEHPFDVNKMVFMDWRDSHLKNNV   269
       * *.***.*.*.*.**..****..**..******** *.

NXS    YLRVNNAKEPTFLYAMPFDRNLVFLEETSLVSRPVLSYME   309
CCS    YLRVKNTKEPTFLYAMPFDRNLVFLEETSLVSRPMLSYME   309
CAPS   ELKERNSRIPTFLYAMPFSSNRIFLEETSLVARPGLGMDD   309
        *. .*.. *********..* .********.** *.  .

NXS    VKRRMVARLRHLGIKVRSVIEEEKCVIPMGGPLPRIPQNV   349
CCS    VKRRMVARLRHLGIKVRSVLEEEKCVITMGGPLPRIPQNV   349
CAPS   IQERMVARLSHLGIKVKSIEEDEHCVIPMGGPLPVLPQRV   349
       .. ******.******.*. *.*.***.****** .**.*

NXS    MAIGGNSGIVHPSTGYMVARSMALAPVLAEAIVKGLGSTR   389
CCS    MAIGGTSGIVHPSSGYMVARSMALAPVLAEAIVESLGSTR   389
CAPS   VGIGGTAGMVHPSTGYMVARTLAAAPVVANAIIQYLSSER   389
       ..***..*.****.******..* ***.*.**.. *.*.*

NXS    MIRGSQLYHRVWNGLWPLDRRCIGECYSFGMETLLKLDLK   429
CCS    MIRGSQLYHRVWNGLWPSDRRRVRECYCFGMETLLKLDLE   429
CAPS   SHSGDELSAAVWKDLWPIERRRQREFFCFGMDILLKLDLP   429
         .*..*   **..*** .**   * ..***..******

NXS    GTRRLFDAFFDLDPKYWQGFLSSRLSVKELAILSLCLFGH   469
CCS    GTRRLFDAFFDVDPKYWHGFLSSRLSVKELAVLSLYLFGH   469
CAPS   ATRRFFDAFFDLEPRYWHGFLSSRLFLPELIVFGLSLFSH   469
       .***.******..*.**.******* . ** ...* **.*

NXS    GSNLTRLDIVTKCPVPLVRLIGNLAIESL   498
CCS    ASNLARLDIVTKCTVPLVKLLGNLAIESL   498
CAPS   ASNTSRLEIMTKGTLPLVHMINNLLQDKE   498
       .** .**.*.** ..***....**  ..
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Fig. 2. Alignment of the predicted amino acid sequences for NXS from *Solanum tuberosum* (accession number AJ272136) and CCS (accession number X76165) and β-LYC (accession number X86221) both from *C. annuum*. The alignment was done using the CLUSTAL program [14]. Identical amino acids are represented by (*), and (.) denotes conserved amino acids.

3.2. The ccs homologous cDNA codes for an enzyme catalyzing neoxanthin formation

Aiming at characterizing the reaction performed by the product of the novel cDNA, we overexpressed the corresponding protein in insect cells using the baculovirus system. However, all attempts to show enzymatic activity in vitro,

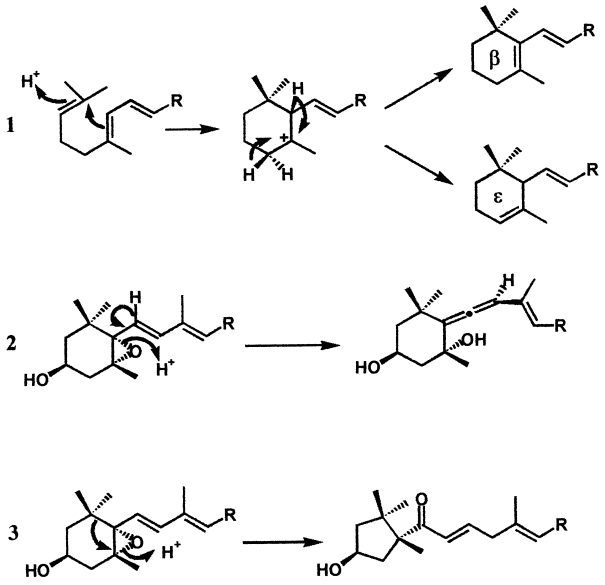

Fig. 3. A model of the reaction mechanisms involved in the catalysis performed by β-LCY, NXS and CCS [12].

used purified violaxanthin as a substrate. Therefore, transient expression studies were performed. Plant protoplasts obtained from *A. thaliana* tissue culture and *N. tabacum* leaves were transformed with the full length cDNA under control of the 35S CaMV promoter by electroporation and PEG, respectively. Both systems yielded the same result with respect to the quality of the carotenoids formed, although they differed in quantity.

Six h after transformation *Nicotiana* protoplasts showed a significant change in the carotenoid complement (Fig. 4). A novel peak appeared in the HPLC trace of transformants which showed a spectrum typical for violaxanthin and neoxanthin, the two being hardly distinguishable by this criterion. However, the difference in the number of in-ring epoxy functions (one in neoxanthin and two in violaxanthin) enabled an unequivocal determination by carrying out an acid catalyzed furanoid rearrangement reaction (not shown). This resulted in a 20 nm hypochromic shift with the newly formed carotenoid, thus demonstrating that neoxanthin was synthesized in transformed cells. The separation system we used allows further resolution of several geometric isomers of violaxanthin and neoxanthin. From the HPLC trace shown it is evident that the state of *cis–trans* isomerism of the product formed is relevant, since only one is predominantly formed. At present, we cannot assign the isomeric state of the newly formed neoxanthin, since the amounts of this carotenoid extractable from the protoplasts used is too small for further analyses. It is known that the overexpression of a single carotenogenic enzyme may lead to alterations of the entire carotenoid content by modifying endogenous biosynthetic activities [10,11]. We therefore examined both the carotenoid pattern as well as the carotenoid content in controls and in transformed protoplasts. There was neither a qualitative nor a quantitative change except the appearance of neoxanthin. Therefore it appears reasonable to conclude that the novel sequence codes for a neoxanthin synthesizing enzyme.

3.3. The NXS is localized in plastids

The biosynthesis of carotenoids is known to occur in plastids. Therefore we checked the localization of the NXS by performing in vitro import studies as described [4]. The electrophoretic mobility of the in vitro translation product obtained corresponded well to the calculated mass of 56 356 Da and the radiolabelled polypeptide was truncated by approximately 8 kDa upon import into plastids (Fig. 5).

4. Discussion

In this work we cloned a *ccs* homologous cDNA from potato, which contains neither capsanthin nor capsorubin.

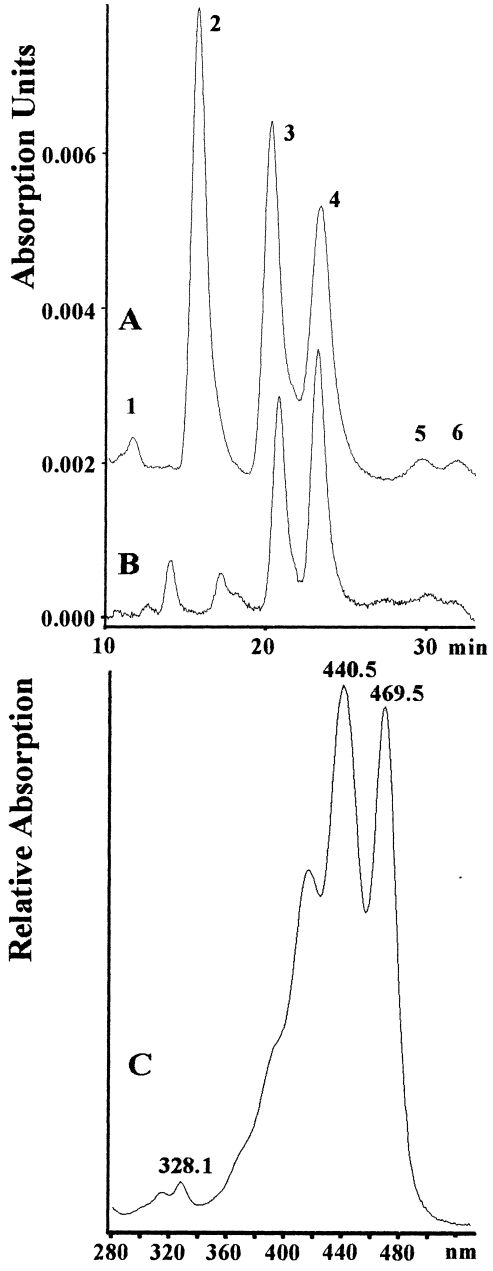

Fig. 4. HPLC trace showing the separation of violaxantin and neoxanthin in (B) control cells and in (A) *nxs* transformed cells. Peaks 1, 2 and 4, neoxanthin isomers; peaks 3, 5 and 6, violaxanthin isomers. The spectrum in (C) represents the newly formed neoxanthin isomer (peak 2 in A) as taken by use of a photodiode array detector in the solvent B of the separation system using a C_{30} reversed phase column.

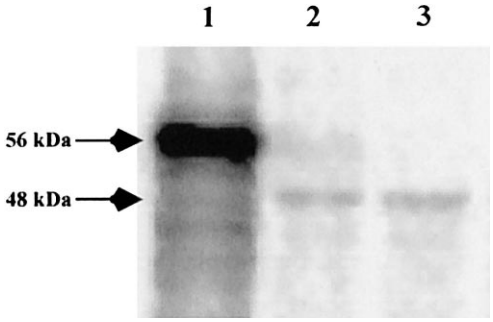

Fig. 5. In vitro plastid import conducted with the NXS translation product. The 56 kDa translation product (lane 1) is imported and truncated by its transit sequence resulting in a mature protein of about 48 kDa. Lane 2, before, and lane 3, after thermolysin digest to remove non-imported proteins.

From theoretical considerations, it has been suggested that the LCY and CCS reactions are very similar ([12], see also [13]). A third reaction catalyzed by NXS belongs to this class of enzymes, acting on carotenoid ring structures (Fig. 3). They all represent acid catalyzed rearrangement reactions, differing in the site of an initial proton attack and the site of proton abstraction to stabilize an intermediate carbenium ion. LCY performs the acid catalyzed cyclization of the carotenoid endgroups from the open chain precursor. Epoxidation of the C(5) double bond opens the route to a variety of metabolites which are produced by an acid catalyzed ring opening of the epoxide to, for example, allen systems (NXS) or by ring contraction to five membered rings (capsanthin).

With the in vivo approach chosen here, we have been able to show that the cloned cDNA codes for a NXS from potato. However, several questions, e.g. as to the nature of the geometric isomerism of substrate and product, cannot yet be answered. Isomerization is particularly important since it has been shown that this feature plays a crucial role in substrate recognition by the cleaving dioxygenase [1]. Looking at steady state changes provoked by transient expression does not allow one to establish such precise precursor–product relationships. This must await the development of an incubation system using the heterologously overexpressed enzyme and defined substrates, and this is currently the subject of further work.

The significant similarity between NXS, CCS and LCY suggests they have a common origin and that CCS and NXS derived from LCY. Recently, a sequence has appeared in the data bases from *Citrus sinensis* (accession number AF169241) termed CCS homologous. It shares at the protein level 69.5% identity with the deduced NXS sequence shown here, however there is no report on function. Considering that *Citrus* does not contain capsanthin, it may therefore represent a NXS from this organism.

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