Phytochemistry 70 (2009) 457–464

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Substrate promiscuity of RdCCD1, a carotenoid cleavage oxygenase from *Rosa damascena*

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ARTICLE INFO

Article history:
Received 4 November 2008
Received in revised form 28 January 2009
Available online 4 March 2009

Keywords:
Rosa damascena
Rose essential oil
(9*Z*)-, (9′*Z*)- and (di-*Z*)-carotenoids
C_{13}-norisoprenoids
Carotenoid cleavage oxygenase
RdCCD1

ABSTRACT

Several of the key flavor compounds in rose essential oil are C_{13}-norisoprenoids, such as β-damascenone, β-damascone, and β-ionone which are derived from carotenoid degradation. To search for genes putatively responsible for the cleavage of carotenoids, cloning of carotenoid cleavage (di-)oxygenase (CCD) genes from *Rosa damascena* was carried out by a degenerate primer approach and yielded a full-length cDNA (*RdCCD1*). The *RdCCD1* gene was expressed in *Escherichia coli* and recombinant protein was assayed for its cleavage activity with a multitude of carotenoid substrates. The *RdCCD1* protein was able to cleave a variety of carotenoids at the 9-10 and 9′-10′ positions to produce a C_{14} dialdehyde and two C_{13} products, which vary depending on the carotenoid substrates. *RdCCD1* could also cleave lycopene at the 5-6 and 5′-6′ positions to produce 6-methyl-5-hepten-2-one. Expression of *RdCCD1* was studied by real-time PCR in different tissues of rose. The *RdCCD1* transcript was present predominantly in rose flower, where high levels of volatile C_{13}-norisoprenoids are produced. Thus, the accumulation of C_{13}-norisoprenoids in rose flower is correlated to the expression of *RdCCD1*.

1. Introduction

Two major species of rose are cultivated for the production of rose oil: *Rosa damascena*, the damask rose, and *Rosa centifolia*, the cabbage rose. Although more than 300 compounds have been identified in rose oil it is the family of rose ketones namely β-damascenone, β-damascone, and β-ionone which contribute considerably to the distinctive scent of rose oil. Even though these compounds exist in less than 1% quantity of rose oil, they make up for slightly more than 90% of the odor content due to their low odor detection thresholds (Ohloff and Demole, 1987). It is assumed that β-damascenone, β-damascone, and β-ionone are derived from carotenoid degradation.

Carotenoids are components of the photosynthetic machinery, intermediates in the biosynthesis of apocarotenoid aroma volatiles, and act as pigments particularly in floral and fruit tissue (Bartley and Scolnik, 1995; Taylor and Ramsay, 2005). The formation of apocarotenoids may result from nonspecific mechanisms, such as lipoxygenase cooxidation or photooxidation (Bosser and Belin, 1994; Yeum et al., 1995; Zorn et al., 2003). A number of apocarotenoids, however, are formed by enzymes capable of cleaving carotenoids regiospecifically.

In recent years, a family of carotenoid cleavage (di)-oxygenases (CCDs) that break carotenoid substrates at different double bond positions has been identified in plants (Bouvier et al., 2005; Kloer and Schulz, 2006; Auldridge et al., 2006b). CCDs exhibit specificity for the double bond that they cleave but many are promiscuous in their substrate choice (Auldridge et al., 2006b). The first carotenoid cleavage oxygenase reported was a 15,15′-oxygenase able to cleave the central carbon 15,15′ double bond of β-carotene giving rise to retinal (Olson and Hayaishi, 1965). In plants, the first *CCD* gene cloned however, was *Zea mays Vp14*, which encodes the prototypic nine-*cis*-epoxycarotenoid dioxygenase (NCED) involved in abscisic acid (ABA) synthesis (Schwartz et al., 1997; Tan et al., 1997; Kalala et al., 2001; Taylor et al., 2005). All NCEDs cleave only 9-*cis* isomers of epoxycarotenoids at the 11,12 position (Tan et al., 2003). CCD7 from Arabidopsis catalyzes the asymmetric cleavage of β-carotene at the 9′,10′ position, producing 10′-apo-β-caroten-10′-al and β-ionone. Interestingly, the Arabidopsis enzyme CCD8 cleaves 10′-apo-β-caroten-10′-al, the cleavage product of CCD7, at the 13,14 position to produce 13-apo-β-carotenone and a C_9 dialdehyde

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doi:10.1016/j.phytochem.2009.01.020

(Schwartz et al., 2004; Auldridge et al., 2006a). The CCD7 and CCD8 enzymes are thought to act in concert to produce a compound involved in the regulation of shoot branching. ZCD from *Crocus sativus* cleaves at the 7,8 (7′,8′) double bonds of zeaxanthin (Bouvier et al., 2003b) yielding one C_{20} and two C_{10} cleavage products that are used for the biosynthesis of crocetin glycosides and safranal, respectively. LCD from *Bixa orellana* was reported to cleave symmetrically at the 5,6 (5′,6′) double bonds of lycopene (Bouvier et al., 2003a), which is the first committed step in the biosynthesis of bixin. The 9,10 (9′,10′) double bonds of all-*trans*- and 9-*cis*-carotenoids as well as epoxycarotenoids are symmetrically cleaved by CCD1 enzymes which produce a C_{14} dialdehyde and two C_{13} products (Schwartz et al., 2001; Simkin et al., 2004a; Ibdah et al., 2006; Rubio et al., 2008). The emission of β-ionone, a C_{13} product from *Petunia hybrida* flowers correlated strongly with the expression levels of *PhCCD1* in corollas (Simkin et al., 2004b). Recently, an additional cleavage activity for CCD1 at the 5,6 (5′,6′) double bonds of lycopene has been reported (Vogel et al., 2008).

The present work describes the isolation of *RdCCD1* from *R. damascena*. To characterize the catalytic activity of RdCCD1, the gene was expressed in *E. coli* and the recombinant protein was assayed for cleavage activity with a variety of carotenoid substrates. In order to study the possible involvement of *RdCCD1* in the synthesis of rose oil flavor compounds, the expression pattern of this gene was determined in various tissues of rose by real-time PCR.

2. Results and discussion

2.1. Cloning of a carotenoid cleavage oxygenase gene from Rosa damascena

Based on the published peptide sequences of carotenoid cleavage oxygenases, we designed forward and reverse degenerate oligonucleotides, which are complementary to the conserved AHPKVDP and MHDFAIT regions, to amplify cDNA fragments from mRNA of *R. damascena* flower. One fragment was obtained, sequenced, and designated as *RdCCD1*. Specific oligonucleotides were designed to obtain the full-length cDNA of *RdCCD1* by RACE-PCR. The nucleotide sequence of *RdCCD1* contains an open reading frame of 1656 bp, and the predicted protein sequence consists of 552 amino acids. The sequence was submitted to Genbank with accession number EU327776. Sequence comparison reveals that *RdCCD1* displays highest similarity (a.a. identity 82%) with *CmCCD1* gene from melon (*Cucumis melo* L.) (Ibdah et al., 2006). A phylogenetic tree shows that *RdCCD1* belong to the *CCD1* cluster (Fig. 1). The predicted RdCCD1 protein contains four highly conversed histidine residues that have been previously described as typical ligands of a non-heme iron co-factor required for (di)-oxygenase activity (Schwartz et al., 1997). In addition, like other *CCD1* genes, no chloroplast transient peptide is found in the *RdCCD1* sequence.

2.2. Functional characterization of RdCCD1

2.2.1. In vivo assay

Due to their extremely low solubility in water, some carotenoid substrates including ζ-carotene, lycopene, and β-carotene are very difficult to use for assay of cleavage activity of CCD *in vitro*. Fortunately, heterologous overexpression of carotenogenesis genes in *E. coli* has proven to be a powerful tool for identifying many of the genes and enzymes involved in carotenoid biosynthesis (Misawa et al., 1995; Hirschberg et al., 1997). We introduced plasmids expressing recombinant *RdCCD1* into *E. coli* strains that accumulate either *cis*-ζ-carotene (Breitenbach and Sandmann, 2005), lycopene, β-carotene, or zeaxanthin (Misawa et al., 1995).

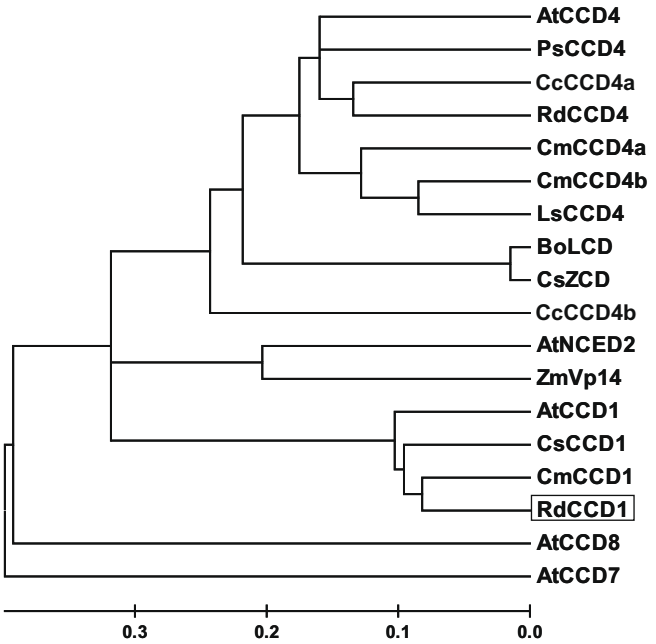

Fig. 1. Phylogenetic tree of the deduced amino acid sequences of carotenoid cleavage oxygenases from *Rosa damascena* and other plant species. Phylogenetic analysis was performed with MEGA program package and the neighbor-joining algorithm (Kumar et al., 2004; http://www.megasoftware.net). Scale bar indicates the phylogenic distances calculated according to the number of differences. The GenBank accession numbers for the sequences are as follows: AtCCD1 (*Arabidopsis thaliana*, NP_191911), CmCCD1 (*Cucumis melo*, DQ269467), RdCCD1 (*Rosa damascena*, ABY47994), CsCCD1 (*Crocus sativus*, AJ132927), AtCCD7 (*Arabidopsis thaliana*, NP182026.1), AtCCD8 (*Arabidopsis thaliana*, NP195007.2), AtNCED2 (*Arabidopsis thaliana*, NP_193569), ZmVp14 (*Zea mays*, AAB62181), CcCCD4b (*Citrus clementina*, DQ309331), BoLCD (*Bixa orellana*, AJ489277), CsZCD (*Crocus sativus*, AJ489276), CmCCD4a (*Chrysanthemum morifolium*, ABY60885), CmCCD4b (*Chrysanthemum morifolium*, BAF36656), LsCCD4 (*Lactuca sativa*, BAE72094), AtCCD4 (*Arabidopsis thaliana*, NP_193652), PsCCD4 (*Pisum sativum*, BAC10552), CcCCD4a (*Citrus clementina*, DQ309330), RdCCD4 (*Rosa damascena*, ABY60886).

Co-expression of *RdCCD1* in strains of *E. coli* accumulating *cis*-ζ-carotene, lycopene, β-carotene, and zeaxanthin led to the development of colourless cultures contrary to the control cultures harbouring an empty vector (Fig. 2A). When *RdCCD1* was overexpressed in *E. coli* producing β-carotene, β-ionone was detected by SPME-GC-MS in the headspace of those cells. However, a small amount of β-ionone (1/100 of that from cells expressing *RdCCD1*) was also detected in the headspace of the control cells which had been transformed with the empty vector. This β-ionone could be derived directly from autoxidation. In addition, geranylacetone was detected in the gas phase of the *E. coli* strain which accumulates *cis*-ζ-carotene and expresses *RdCCD1*. β-Ionone and geranylacetone were produced by cleavage of the 9,10 and 9′,10′ positions of β-carotene and *cis*-ζ-carotene, respectively (Fig. 2B).

Geranylacetone, 6-methyl-5-hepten-2-one (MHO), *cis*-pseudoionone, and *trans*-pseudoionone were detected in the headspace of *E. coli* strain producing lycopene and expressing *RdCCD1* (Fig. 2C). Geranylacetone (1/10 of pseudoionone level) is probably a breakdown product of phytoene, an intermediate in lycopene biosynthesis (Fig. 2B). This phenomenon was also found in the study with *CmCCD1* (Ibdah et al., 2006). Interestingly, the level of MHO detected was 2.5 times that of pseudoionone. MHO is produced by cleavage of the 5,6 and 5′,6′ positions of lycopene (Fig. 2B). This result indicates that the rose CCD1 enzyme, like the Arabidopsis, maize, and tomato CCD1, cleaves lycopene at 5,6 (5′,6′) bonds to generate MHO (Vogel et al., 2008). Low levels of geranylacetone, MHO, pseudoionone, and β-ionone were identified

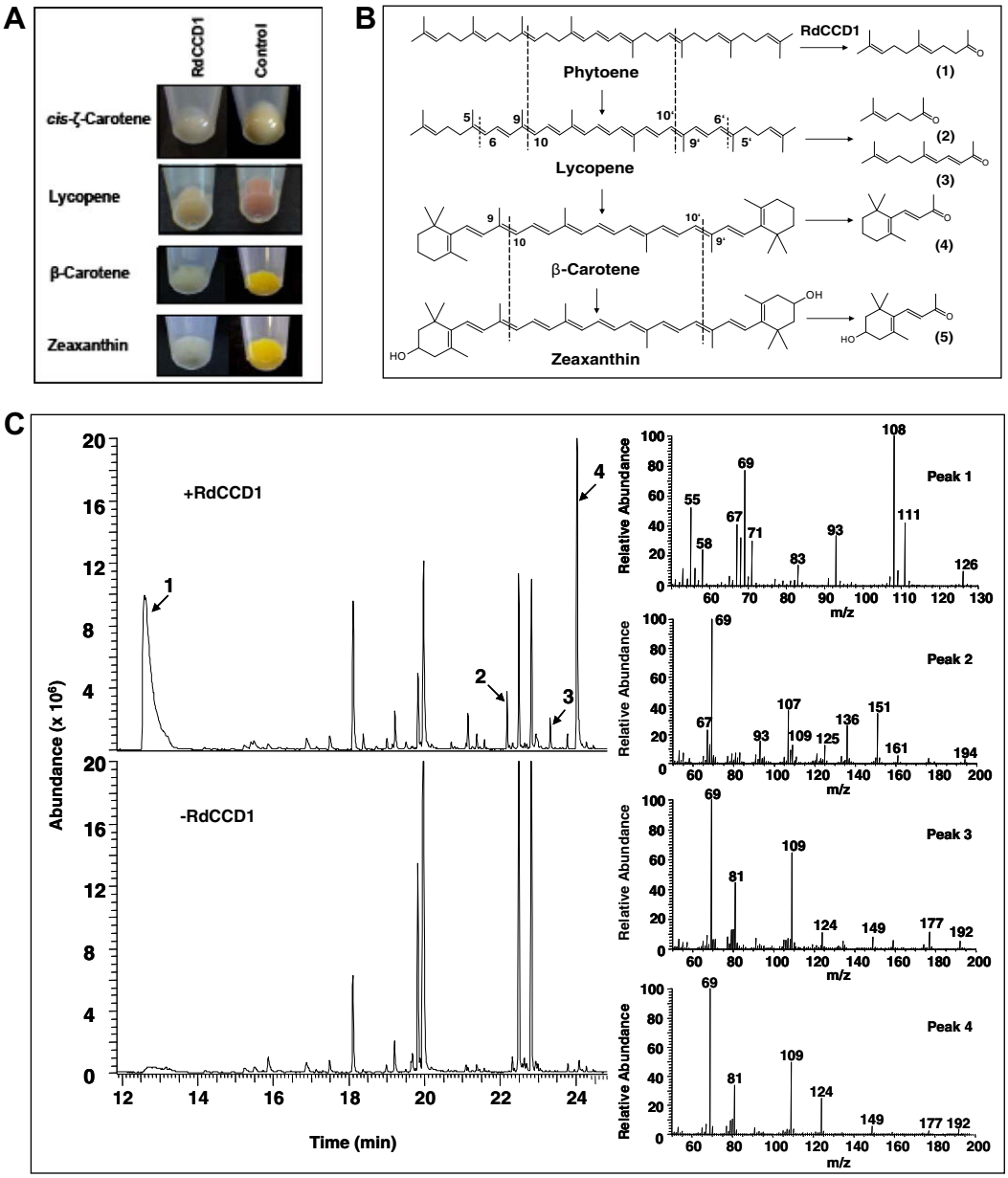

Fig. 2. Functional analysis of RdCCD1 protein. (A) *Escherichia coli* strains engineered to accumulate *cis*-ζ-carotene, lycopene, β-carotene, and zeaxanthin were cotransformed with the pGEX-*RdCCD1* (*RdCCD1*) and pGEX-4T1 empty vector (Control). (B) Carotenoid biosynthetic pathway of *Erwinia* species and the proposed sites of RdCCD1 bond cleavage as well as the volatiles generated. Carotenoid substrates (left) are oxidatively cleaved to yield the volatiles (right): geranylacetone (1), 6-methyl-5-hepten-2-one (MHO, 2), *trans*-pseudoionone (3), β-ionone (4), and 3-hydroxy-β-ionone (5). Dashed lines indicate sites of RdCCD1 cleavage. (C) GC-MS analyses of the bacterial headspace of *E. coli* strain accumulating lycopene. Top panel, *RdCCD1* expressing cells; bottom panel, control cells. The mass spectra of peaks 1, 2, 3, and 4 yield fragmentation patterns identical to those of MHO, geranylacetone, *cis*-pseudoionone, and *trans*-pseudoionone, respectively.

in the gas phase of the *E. coli* strain accumulating zeaxanthin and expressing *RdCCD1*. 3-Hydroxy-β-ionone could not be found in the headspace probably due to its low volatility and small quantity. However, by HPLC-MS, it was possible to detect carotenoid cleavage products in organic extracts of cell medium from induced *E. coli* cultures expressing *RdCCD1* in zeaxanthin-accumulating *E. coli* cells. These extracts showed low levels of C_{14} dialdehyde and 3-hydroxy-β-ionone (data not shown), indicating a symmetrical cleavage of the substrate at the 9,10 and 9′,10′ positions. Geranylacetone, MHO, pseudoionone, and β-ionone are breakdown products of phytoene, lycopene, and β-carotene, respectively, which are intermediates in zeaxanthin biosynthesis (Misawa et al., 1995). Thus, obviously due to the degradation of its precursors, only a small quantity of zeaxanthin is finally cleaved to yield a low level of 3-hydroxy-β-ionone.

2.2.2. In vitro assay

To determine whether *RdCCD1* encodes a functional CCD, the gene was cloned into a glutathione S-transferase fusion vector for expression in *E. coli*. The recombinant protein was assayed for cleavage activity with a variety of carotenoid substrates. They can be grouped into four classes, (i) all *trans*-substrates (Fig. 3A), (ii) (9*Z*)-substrates (Fig. 3B), (iii) violaxanthin isomers (Fig. 3C), and (iv) apocarotenoids (Fig. 4A). After incubation, the products were separated by thin layer chromatography (TLC) and visualized by UV-visible spectroscopy or directly applied to GC-MS analyses

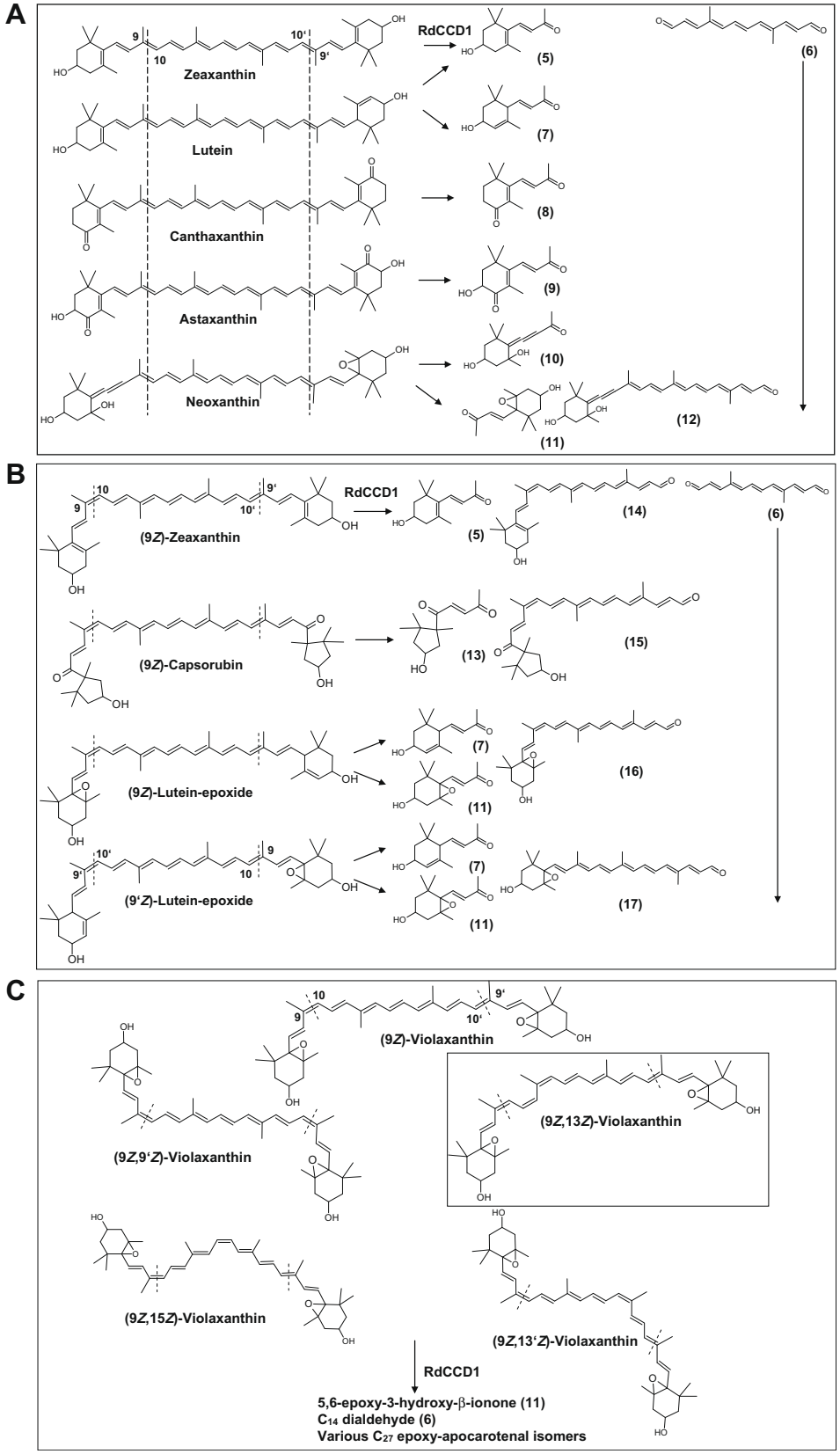

Fig. 3. Carotenoid substrates and their degradation products generated by RdCCD1. (A) all *trans*-carotenoid substrates; (B) (9*Z*)-carotenoid substrates; (C) Violaxanthin isomers. The numerals correspond to compounds 3-hydroxy-β-ionone (5), 4,9-dimethyldodeca-2,4,6,8,10-pentaene-1,12-dial (C_{14}-dialdehyde, 6), 3-hydroxy-α-ionone (7), 4-oxo-β-ionone (8), 3-hydroxy-4-oxo-β-ionone (9), grasshopper ketone (10), 5,6-epoxy-3-hydroxy-β-ionone (11), (all-*E*,3*S*,5*R*,6*R*)-3,5-dihydroxy-6,7-didehydro-10′-apo-β-caroten-10′-al (C_{27} allenic-apocarotenal, 12), 1-((1*R*,4*S*)-4-hydroxy-1,2,2-trimethylcyclopentyl)-2-pentene-1,4-dione (13), (9*Z*,3*R*)-3-hydroxy-10′-apo-β-caroten-10′-al ((9*Z*)-apo-10′-zeaxanthinal, 14), (9*Z*,3*S*,5*R*)-3-hydroxy-10′-apo-κ-caroten-10′-al ((9*Z*)-capsylaldehyde, 15), (9*Z*,3*S*,5*R*,6*S*)-5,6-epoxy3-hydroxy-10′-apo-β-caroten-10′al ((9*Z*)-apo-10′-violaxanthal, 16), and (all-*E*,3*S*,5*R*,6*S*)-5,6-epoxy-3-hydroxy-10′-apo-β-caroten10′-al ((all-*E*)-apo-10′-violaxanthal, 17). Dashed lines indicate sites of *RdCCD1* cleavage.

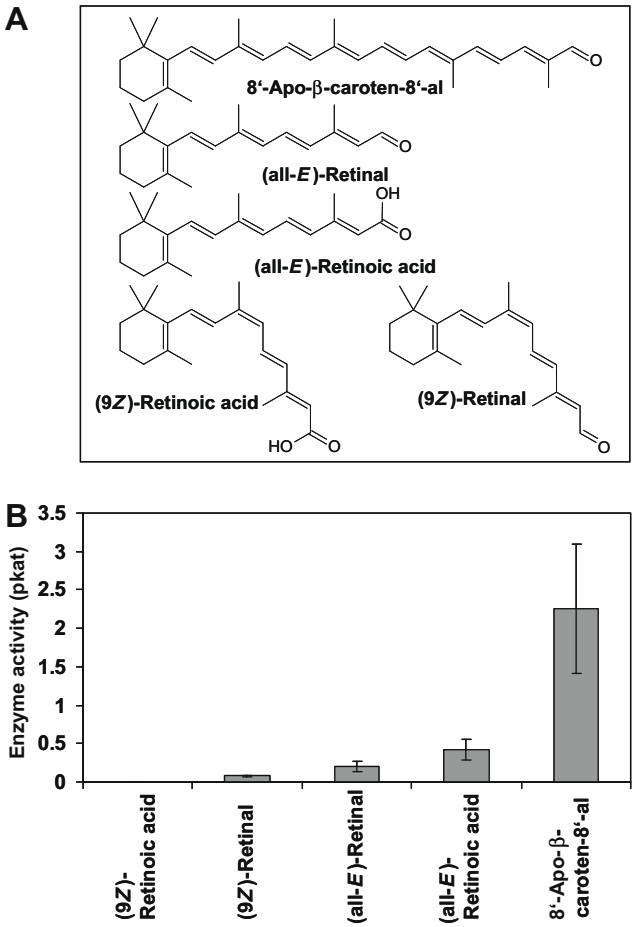

Fig. 4. The catalytic activity of RdCCD1 protein towards various apocarotenoid substrates. (A) Structures for the apocarotenoid substrates examined. (B) Catalytic activity with substrate (9*Z*)-retinoic acid, (9*Z*)-retinal, (all-*E*)-retinal, (all-*E*)-retinoic acid, and 8′-apo-β-caroten-8′-al. Values are the means ± SEM of three separate determinations performed at 30 °C in a 1% β-octylglucoside solution containing 6.25 μg of substrates.

in the case of apocarotenoid substrates. The results showed that RdCCD1 could cleave all the substrates examined, except for (9*Z*)-retinoic acid and (9*Z*)-violaxanthin-diacetate. The cleavage products formed by RdCCD1 were further characterized by GC-MS and LC-MS. A variety of C_{13} products (Fig 3A, B, and C), resulting from cleavage at the 9-10 and the 9′-10′ positions, were identified by GC-MS (Supplementary material Fig. S1). The central C_{14} dialdehyde cleavage product resulting from symmetrical cleavage at the 9-10 and the 9′-10′ positions (Fig 3A, B and C) was first separated by TLC and then characterized by LC-MS. Asymmetric cleavage leading to the formation of C_{27} apocarotenoids was found in the reaction mixtures containing neoxanthin and (9*Z*)-substrates (Fig. 3A, B, and C). Various C_{27} products were identified by LC-MS.

Taken together, the results of the *in vivo* and *in vitro* assays indicate that RdCCD1 is a carotenoid cleavage oxygenase which could cleave the 9,10 (9′,10′) double bonds of multiple carotenoid substrates to produce a C_{14} dialdehyde and two C_{13} products. RdCCD1 is able to release geranylacetone, pseudoionone, β-ionone, 3-hydroxy-α-ionone, 3-hydroxy-β-ionone, grasshopper ketone, 5,6-epoxy-3-hydroxy-β-ionone, 4-oxo-β-ionone, 3-hydroxy-4-oxo-β-ionone, and 1-((1*R*,4*S*)-4-hydroxy-1,2,2-trimethylcyclopentyl)-2-pentene-1,4-dione. Among these, β-ionone and grasshopper ketone play an important role in the synthesis of rose oil flavor compounds. RdCCD1 could also cleave the 5,6 (5′,6′) double bonds of lycopene to produce MHO. In addition, RdCCD1 is also able to cleave some carotenoids only at one end, releasing C_{27} apocarotenoids.

2.3. Substrate promiscuity of RdCCD1

In total, 23 substrates were used to analyse RdCCD1 activity, including linear and cyclic carotenoids, symmetrical and asymmetrical carotenoids, all-*trans*-carotenoids and 9-*cis* carotenoids as well as carotenoids containing more than one *cis* double bonds. RdCCD1 is able to cleave a variety of substrates as described above. This indicates that the rose CCD1 enzyme, like the Arabidopsis CCD1 (Schwartz et al., 2001), tomato CCD1 (Simkin et al., 2004a), melon CCD1 (Ibdah et al., 2006) and maize CCD1 (Vogel et al., 2008), shows broad substrate tolerance and produces numerous products.

2.4. The cleavage activity of RdCCD1

When carotenoids like zeaxanthin, canthaxanthin, and astaxanthin containing symmetrical moiety at both ends were used as substrates for RdCCD1, the C_{14} dialdehyde was a major product resulting from symmetrical cleavage at the 9-10 and 9′-10′ positions. Interestingly, a C_{27} product formed by a single cleavage was only detected when substrates contain different moieties at their ends. Asymmetric cleavage leading to the formation of C_{27} allenic-apocarotenal (12, Fig. 3A) was found in the reaction containing neoxanthin. The level of 5,6-epoxy-3-hydroxy-β-ionone (11, Fig. 3A) detected in this reaction was 4 times that of grasshopper ketone (10, Fig. 3A). This result revealed that RdCCD1 does not cleave well at a position adjacent to an allenic bond but prefers conjugated double bonds. However, although lutein contains asymmetrical moieties at the ends, the same levels of 3-hydroxy-β-ionone (5, Fig. 3A) and 3-hydroxy-α-ionone (7, Fig. 3A) were detected, indicating the same cleavage efficiency at 9-10 and 9′-10′ positions. Asymmetric cleavage was also observed in the reactions with (9*Z*)-substrates. (9*Z*)-C_{27}-products were detected in the reactions containing (9*Z*)-zeaxanthin, (9*Z*)-capsorubin, (9*Z*)-lutein-epoxide, and (9*Z*)-violaxanthin indicating that RdCCD1 did not cleave well at a 9,10-*cis* double bond. Unexpectedly, instead of a (9*Z*)-C_{27}-product, the all *trans*-C_{27}-product was detected by LC-MS analysis when (9′*Z*)-lutein-epoxide was used as a substrate (Fig. 3B). This result corresponded to that from GC-MS analysis, which showed that more 3-hydroxy-α-ionone (7, Fig. 3B) than 5,6-epoxy-3-hydroxy-β-ionone (11, Fig. 3B) was formed no matter whether (9*Z*)-lutein-epoxide or (9′*Z*)-lutein-epoxide was used as a substrate. The results indicated that RdCCD1 cleaves a *cis*-double bond adjacent to a moiety with 3-hydroxy-α-ionone even better than a *trans*-double bond adjacent to a moiety with a 5,6-epoxy-3-hydroxy-β-ionone structure. Taken together, the enzyme activity of RdCCD1 depends not only on *cis* or *trans* double bonds but also on the moieties at the ends. However, no cleavage products were detected when (9*Z*)-violaxanthin-diacetate was used as a substrate. Thus, acylation of xanthophylls can protect them from being degraded by CCD1.

The cleavage activity of RdCCD1 was also affected by the positions of *cis* double bonds. Various violaxanthin isomers: (9*Z*)-violaxanthin, (9*Z*,9′Z)-violaxanthin, (9*Z*,13*Z*)-violaxanthin, (9*Z*,15*Z*)-violaxanthin, and (9*Z*,13′*Z*)-violaxanthin were applied in RdCCD1 enzyme activity tests (Fig. 3C). The amounts of C_{14} and C_{27} products were determined by LC-MS. The largest ratio of C_{27}-product/C_{14}-product was found in the reaction containing (9*Z*,13*Z*)-violaxanthin in contrast to the reaction containing (9*Z*,9′*Z*)-violaxanthin, indicating that the closer the second *cis* double bond is to a 9-*cis* double bond, the lower is the cleavage activity observed. Generally, the accumulation of a C_{27} product implies that the C_{40} carotenoid is a better substrate for RdCCD1 than the C_{27} apocarotenal.

Various apocarotenoids were also used for RdCCD1 activity assays, including (all-*E*)-8′-apo-β-caroten-8′-al (C_{30}), (all-*E*)-retinoic acid (C_{20}), (all-*E*)-retinal (C_{20}), (9*Z*)-retinal (C_{20}), and (9*Z*)-retinoic acid (C_{20}, Fig. 4A). The best cleavage activity was observed in the reaction containing (all-*E*)-8′-apo-β-caroten-8′-al (5, Fig. 4B), whereas the reaction containing (9*Z*)-retinoic acid showed almost no cleavage activity (1, Fig. 4B). However cleavage activity was much higher when (all-*E*)-retinoic acid was used as a substrate (4, Fig. 4B). This result supports that RdCCD1 cleaves a *trans* double bond at the 9,10 position more efficiently than a *cis* double bond. This conclusion was also confirmed in the reactions containing (all-*E*)-retinal and (9*Z*)-retinal. Moreover, RdCCD1 cleaved C_{30}- better then C_{20}-apocarotenoid.

2.5. Spatial distribution of RdCCD1 gene transcript in the rose plant

The expression pattern of *RdCCD1* was examined in various rose organs by real-time PCR (Fig. 5). *RdCCD1* was detected in all organs examined with the highest level in flower. The expression level in flower was 2-fold higher than the level found in leaf, 5-fold higher than that in stem, and 8-fold higher than that in root. β-Damascenone, a presumed metabolite of grasshopper ketone, β-damascone, and β-ionone are key flavor compounds in rose oil which is produced from rose flower (Demole et al., 1970; Ohloff and Demole, 1987). The expression of *RdCCD1* correlates with the accumulation of high levels of volatile C_{13}-norisoprenoids in rose flower and therefore affects the quality of rose oil. Similarly in *Petunia hybrida* flowers the expression levels of *PhCCD1* in corollas correlated strongly with the emission of β-ionone (Simkin et al., 2004b). During tomato fruit development *LeCCD1B* is also found to be implicated in the C_{13}-norisoprenoid synthesis (Simkin et al., 2004a). These data indicate that CCD1 plays a significant role in volatile C_{13}-norisoprenoid synthesis in plants.

3. Conclusion

The flowers of *R. damascena* are utilized for the production of rose essential oil, in which volatile C_{13}-norisoprenoids significantly contribute to the odor although they occur only in trace amounts. Among them, β-damascenone was the first constituent to be identified in the essential oil of *R. damascena* Mill (Demole et al., 1970). However, β-damascenone is neither produced nor released from the flowers of *R. damascena* Mill. It has been suggested that β-damascenone is produced from progenitors during the steam distillation of the rose flowers. Furthermore, it was postulated that the progenitors of β-damascenone are produced by enzymatic cleavage of carotenoids (Isoe et al., 1973; Ohloff et al., 1973). One of the progenitors of β-damascenone is grasshopper ketone, which is a primary degradation product from neoxanthin (Suzuki et al., 2002). In addition to β-damascenone, β-ionone is also a key flavor compound that contributes to the distinctive scent of rose oil and is a product of the oxidative cleavage of β-carotene (Winterhalter and Rouseff, 2002).

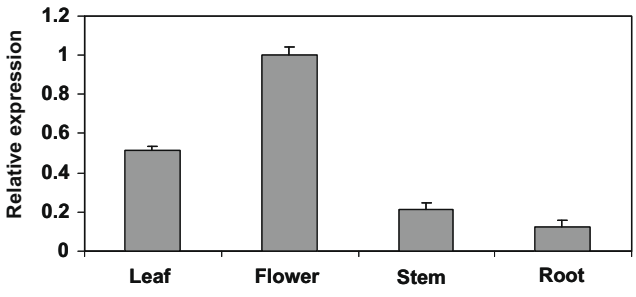

Fig. 5. Spatial distribution of *RdCCD1* gene transcript in the rose plant. Quantitative real-time RT-PCR analysis was performed using *RdCCD1* and *Interspace* gene specific primers, the latter used as internal control for normalization. Total RNA was extracted from mature leaves, whole flowers (full bloom), stems, and roots of a potted rose plant (*R. damascena*). Values are means ± SEM of three different evaluations carried out with two sets of cDNAs.

We have isolated a *CCD* gene from *R. damascena* (RdCCD1) which was functionally expressed in *E. coli* and used for *in vivo* and *in vitro* assays. The RdCCD1 protein was able to cleave a variety of carotenoids (and also apocarotenoids) at the 9-10 and 9′-10′ positions to produce a C_{14} dialdehyde and two C_{13} products, which vary depending on the carotenoid substrates. We clearly demonstrated that β-ionone is produced by RdCCD1 from β-carotene and grasshopper ketone is formed by RdCCD1 from neoxanthin. It is assumed that grasshopper ketone is subsequently reduced to grasshopper alcohol (3-hydroxy-7,8-didehydro-β-ionol) and finally glucosylated. Glucosides of 3-hydroxy-7,8-didehydro-β-ionol have been identified as precursors of β-damascenone because they yield the norisoprenoid upon heating under acidic condition similar to steam distillation (Suzuki et al., 2002). Thus, RdCCD1 is most likely involved in the formation of two key constituents of rose essential oil. Besides, RdCCD1 could cleave lycopene at the 5-6 and 5′-6′ positions to produce MHO. This ketone and its corresponding secondary alcohol have also been found as trace components in the essential oil produced from *R. damascena* (Bayrak and Akgül, 1994). Additional degradation products formed by RdCCD1 from other carotenoids or xanthophylls have not yet been described in *R. damascena* flowers but could be bound to glucose and thus escape from detection (Suzuki et al., 2002).

Real-time PCR showed that *RdCCD1* transcript was present predominantly in rose flower, indicating that *RdCCD1* is involved in the biosynthesis of C_{13}-norisoprenoids in this tissue due to the high activity of the encoded protein. The cloning and functional characterization of *RdCCD1*, an essential gene for carotenoid degradation now provides the foundation for metabolic engineering of rose oil to increase the content of norisoprenoids and the biotechnological production of norisoprenoid aroma chemicals.

4. Experimental

4.1. Cloning of Rosa damascena CCD1 cDNA

Total RNA was isolated from flower of *R. damascena* by CTAB extraction (Liao et al., 2004). The first-strand cDNAs were synthesized from 1 μg of total RNA using Superscript III RTase (Invitrogen) and a GeneRacer oligo-dT primer (5′-GCT GTC AAC GAT ACG CTA CGT AAC GGC ATG ACA GTG $T_{(18)}$-3′). The cDNA fragments of *CCD* genes were amplified by PCR with the cDNA template of flower of *R. damascena* and a set of primers: 5′-GCN CAY CCN AAR GTN GAY CC-3′ (forward) and 3′-CAY GAY TTY GCN ATH ACN GA-5′ (reverse) designed by common sequences that have been reported (Watillon et al., 1998; Schwartz et al., 1997, 2001, 2004; Bouvier et al., 2003b; Ohmiya et al., 2006; Ibdah et al., 2006; Mathieu et al., 2005; Agustí et al., 2007). The amplified cDNAs were cloned into pGEM-T vector (Promega) and their sequences were determined. After sequencing, a cDNA clone displaying high sequence similarity to other plant CCD genes was obtained and designated as *RdCCD1*. The full-length cDNA clone for *RdCCD1* was obtained by RACE-PCR using GeneRacer oligo-dT primer and gene specific primers.

4.2. Real-time RT-PCR analysis

Total RNA was extracted from mature leaves, whole flowers (full bloom), stems, and roots of a potted rose plant (*R. damascena*,

50 cm high) using the CTAB extraction procedure (Liao et al., 2004). RNA samples were treated with RNAse free DNAse I (Fermentas) for 1 h at 37 °C. First strand cDNA synthesis was performed in duplicate in a 20 μl reaction volume, with 1 μg of total RNA as the template, random primer (random hexamer, 100 pmol), and M-MLV reverse transcriptase (200 U, Invitrogen) and according to the manufacturer's instructions. For each real-time PCR reaction, 2 μl of cDNA were used. Real-time PCR was performed on an StepOnePlus System (Applied Biosystems, USA) using SYBR Green PCR Master MIX (Applied Biosystems, USA). To monitor dsDNA synthesis data was analyzed with ABI StepOne Software v2.0. A relative quantification of gene expression was performed using the *Interspacer* gene as a reference. Primers for the amplification of *Interspacer* gene were 5′-ACC GTT GAT TCG CAC AAT TGG TCA TCG-3′ (forward) and 5′-TAC TGC GGG TCG GCA ATC GGA CG-3′ (reverse). The primers used for the target gene *RdCCD1* were 5′-GAA GCC GAG CAA GGG CGT CAC CTC CAC ACT-3′ (forward) and 5′-TTG GGG TTG GGG CCA ACC CTT ACA AAC TCT-3′ (reverse). All reactions were run three times with two sets of cDNAs. The thermal cycling conditions consisted of 50 °C for 2 min followed by an initial denaturation step at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, then 60 °C for 1 min. The specificity of the PCR amplification was checked with a melting curve analysis following the final step of the PCR. For each sample, threshold cycles (Ct, cycle at which the increase of fluorescence exceeded the threshold setting) were determined. Relative expression ratio was calculated and normalized using the *Interspace* gene (Pfaffl, 2001).

4.3. Cloning of RdCCD1 into carotenoid accumulating E. coli strains

The full-length open reading frame of *RdCCD1* was amplified by RT-PCR from first-strand cDNA synthesized from total RNA of rose flower with Phusion DNA polymerase (New England Biolabs) and the following primer sequences: 5′-AAA GGA TCC ATG GCG GAG GTG GTC GAG AAG-3′ (forward) and 5′-AAA GAA TTC TTA GAA CTT TGC TTG TTC TTG C-3′ (reverse). The PCR products and the pGEX-4T1 vector were double digested with the restriction enzymes (*BamH*I and *Eco*RI, the recognition sequences are underlined), and then ligated. After sequencing the plasmids pGEX-*RdCCD1* and pGEX-4T1 empty vector (negative control) were transformed into *E. coli* strains engineered to accumulate *cis*-ζ-carotene (Breitenbach and Sandmann, 2005), lycopene, β-carotene, and zeaxanthin (Misawa et al., 1995).

4.3.1. Carotenoid and product analyses

An overnight culture of 2 ml was used to inoculate 200 ml of LB medium containing the appropriate antibiotics. The cultures were incubated at 37 °C with gentle shaking (125 rpm) till OD_{600} of 0.6. After adding 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG), the cultures were grown at 16 °C for additional 20 h and gently shaken (125 rpm). Carotenoids and products were extracted from bacterial cells or growth medium as described (Schwartz et al., 2001), and then analysed by HPLC.

HPLC separations were performed on a reverse phase column (Grom Sil 100 ODS PE, 5 μm, 250 × 4 mm). The mobile phases used were water containing 0.1% formic acid (A), 100% acetonitrile containing 0.1% formic acid (B), and isopropanol (C). The gradient was as follows: 0–20 min, 0–50% B; 20–25 min, 50–100% B; 25–35 min, 100% B to 70% B/30% C, hold for 10 min; 70% B/30% C to 100% B in 3 min; 100% B to 100% A in 5 min, then hold for 7 min. The flow rate was 1 ml min^{-1} and the detection wavelength was 350 nm.

4.3.2. Determination of volatiles from bacterial headspace

An overnight culture (0.5 ml) was used to inoculate 20 ml of LB medium containing the appropriate antibiotics in 200 ml flasks. The flasks were incubated at 37 °C with gentle shaking (125 rpm) till OD_{600} of 0.6. After adding 0.2 mM IPTG, 5 ml of cell culture was transferred to a tightly closed 40 ml tube. The tubes were incubated at 16 °C for 20 h and gently shaken (125 rpm). A SPME fiber (65 μm polydimethylsiloxane-divinylbenzene, Supelco) was introduced into the vial through a septum and the headspace volatiles were allowed to be adsorbed by the fiber at 45 °C for 30 min. Subsequently the SPME fiber was introduced into the GC. The volatile compounds collected from the headspace were analyzed on a Thermo Finnigan Trace DSQ mass spectrometer coupled to a 0.25 μm BPX5 20 M fused silica capillary column with a 30 m × 0.25 mm inner diameter. He (1.1 ml min^{-1}) was used as a carrier gas. The injector temperature was 250 °C, set for splitless injection. The temperature program was 40 °C for 1 min, 40–60 °C at a rate of 2 °C min^{-1}, and 60–325 °C at 10 °C min^{-1}. The ion source temperature was 250 °C. Mass range was recorded from *m/z* 50–300 and spectra were evaluated with the Xcalibur software version 1.4 supplied with the device.

4.4. Protein expression and enzyme assays in vitro

The plasmids pGEX-*RdCCD1* and pGEX-4T1 empty vector (negative control) were transformed into *E. coli* BL21 DE3pLysS for protein expression. A 2 ml overnight culture was used to inoculate a 100 ml culture in LB medium containing 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloroamphenicol. Cultures were grown at 37 °C until an OD_{600} of 0.6 was reached. Expression of the protein was induced by the addition of 0.2 mM IPTG, and the cultures were grown at 16 °C for an additional 20 h. The crude extract was prepared and enzyme activity assay was performed as described (Schmidt et al., 2006). The assay products were partitioned into ethyl acetate and analyzed by thin-layer chromatography as described (Schwartz et al., 2001). The mass spectra for C_{14}, C_{17}, and C_{27} compounds were obtained by LC-MS analysis as described (Schmidt et al., 2006). For gas chromatography-mass spectrometer of the C_{13} compounds, a Thermo Finnigan Trace DSQ mass spectrometer coupled to a Thermo Finnigan Trace GC with a split injector (1:10) and a 0.25 μm BPX5 20 M fused silica capillary column with a 30 m × 0.25 mm inner diameter was used. The oven temperature was held at 40 °C for 1 min and then increased to 240 °C at 3 °C min^{-1} intervals, with a helium flow rate of 1.1 ml min^{-1}. The EI-MS ionization voltage was 70 eV (electron impact ionization), and the ion source temperature was 230 °C. Mass range was recorded from 45 to 450 *m/z* and spectra were evaluated with the Xcalibur software version 1.4.

Acknowledgements

This study was supported by grants from the Federal Ministry for Economy and Technology of Germany (BMWi) via the AiF ZU-TECH program 110 ZN and 243 ZN as well as from OTKA K 60121 and K 76176 (Hungarian National Research Foundation). C. Christiansen from WILD Flavors is thanked for providing carotenoid samples.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.01.020.

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