Chapter 9

# Biosynthesis of $C_{13}$-Norisoprenoids in *Vitis vinifera*: Evidence of Carotenoid Cleavage Dioxygenase (CCD) and Secondary Transformation of Norisoprenoid Compounds

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Although the chemistry and occurrence of volatile norisoprenoids in plants is well studied little is known about their biosynthesis. This chapter gives an overview on the biosynthesis of $C_{13}$-norisoprenoids in grape berries, main class of norisoprenoids in *Vitis vinifera*. Experiments supportive for an apo-carotenoid pathway, the evidence of a carotenoid cleavage dioxygenase (CCD) from *Vitis vinifera* giving rise to $C_{13}$-norisoprenoids and the secondary transformations of $C_{13}$-norisoprenoids by grape cell culture are discussed.

Several $C_{13}$-norisoprenoids have been detected in grape berries and leaves (*1–4*). Some of them, such as β-damascenone, β-ionone, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), Riesling acetal, vitispirane, and actinidol are potent aroma contributors in both red and white wines (*2*, *5–7*). In contrast to terpenes, $C_{13}$-norisoprenoids occur mainly as glycoconjugates in grape berry and wine leaves (*4*, *8*). This chapter discusses the biogenesis of $C_{13}$-norisoprenoids in grape berries and is presented in the following order: (i) arguments in favor of an apo-carotenoid pathway, (ii) evidence of a carotenoid cleavage dioxygenase (CCD), and (iii) secondary transformations of $C_{13}$-norisoprenoids.


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## Arguments in Favor of an Apo-Carotenoid Pathway

Before the evidence of the existence of a CCD in *Vitis vinifera* (*9*) there were several assumptions in favor of an apo-carotenoid pathway to explain the origin of $C_{13}$-norisoprenoids in grapes as discussed below:

### Relation between the Levels of $C_{13}$-Norisoprenoids and Carotenoids during Grape Berry Maturation

During grape berry ripening from Muscat *cv*. the levels of $C_{13}$-norisoprenoids and carotenoids were found to change in opposite directions (*10*). A decline in carotenoid levels (β-carotene and xanthophylls) was observed from veraison to maturity, with the major reduction occurring at veraison. During the same period the levels of $C_{13}$-norisoprenoids increased with increasing maturity. In this study only the levels of $C_{13}$-norisoprenoids occurring as glycoconjugates were considered since their free counterparts were negligible in grape berries.

### Relation between Xanthophyll Cycle and $C_{13}$-Norisoprenoid Synthesis

Interconversions between different carotenoids taking part in the xanthophyll cycle under light and shaded conditions provided supplementary clues on the origin of norisoprenoids (*11*). Under light conditions, higher levels of 3-oxo-α-ionol, 3-hydroxy-7,8-dihydro-β-ionone and 3-hydroxy-7,8-dihydro-β-ionol were observed. This increase may be attributed to the non-epoxy xanthophylls, like zeaxanthin, lutein since their synthesis is favored under light. On the contrary, epoxyxanthophylls synthesis is favored under shade conditions producing higher levels of violaxanthin and neoxanthin. Higher levels of 3-hydroxy-5,6-epoxy-β-ionol, 3-hydroxy-7,8-dehydro-β-ionol and 4,5-dihydro-vomifoliol were also observed suggesting structural relationships between the norisoprenoids and their parent carotenoids. The exposure of grape berries to sunlight during maturation increased the level of $C_{13}$-norisoprenoids and decreased that of carotenoids compared to shaded berries. This supports the argument for an apo-carotenoid pathway for the synthesis of $C_{13}$-norisoprenoids (*11–13*). Furthermore increases in the levels of glycoconjugated TDN and Riesling acetal in white Riesling grapes were also observed when sunlight exposure greater than 20% of full sun exposure was applied beginning at veraison (*14*).

### Relation between the Stereochemistry of $C_{13}$-Norisoprenoids and Carotenoids

The comparison of the asymmetric centers of $C_{13}$-norisoprenoids with those of the putative grape carotenoids supported the hypothesis that carotenoids are precursors of norisoprenoids. Indeed, formation of megastigmane-3,9-diol and 3-oxo-α-ionol from the ε-ring of lutein was suggested since the stereochemistry


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of carbons-3 and -6 was maintained (*15*). Similarly formation of 3-hydroxy-β-damascone and β-damascenone from neoxanthin was proposed because of the maintenance of the stereochemistry of carbon-3 (*15–17*).

## $^{13}$C-Labelling Experiment

$^{13}$C-labelling experiments were performed to introduce $^{13}CO_2$ into berries during berry ripening in order to examine a possible apo-carotenoid pathway through the analysis of carbon isotopic ratios in carotenoids and norisoprenoids (*15*, *18*) (Figure 1).

$^{13}CO_2$ labeling of berries was performed before veraison since carotenoid synthesis in grape berries occurs mainly prior to veraison (*10*). Grape bunches from Syrah cv. were hermetically maintained in the bags and were fed with $^{13}CO_2$ from 8 a.m to 3 p.m each day from June 16 to July 26. The feeding started 16 days following berry set, lasted for 40 days and ended 6 days before veraison. At the end of each $^{13}CO_2$ feeding, the bags were flushed with air. To trap humidity and hence avoid fungal development on the grapes the water in the circuit was cold-trapped. Samplings were done at two moments, 6 days before veraison (August 01) and at maturity (September 19). Levels and isotopic ratios of $^{13}C/^{12}C$ (δ, ‰) of β-carotene and xanthophylls from labeled and control berries were determined. In parallel the same determination was performed for 3-oxo-α-ionol, one of the major glycoconjugated $C_{13}$-norisoprenoid in grape.

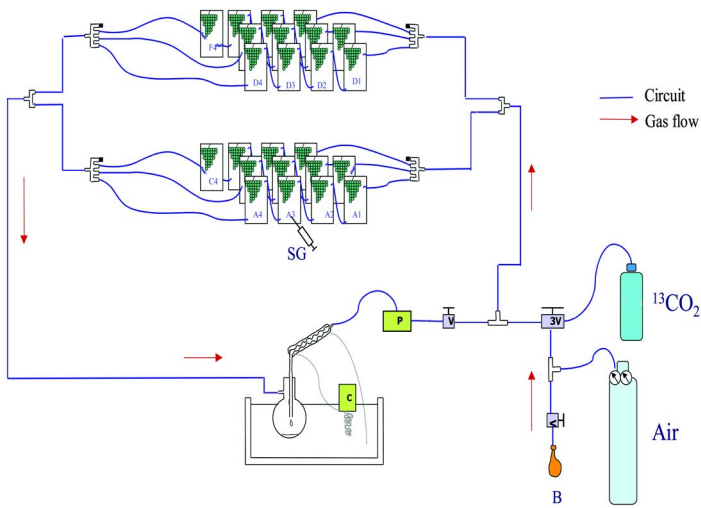


*Figure 1. $^{13}CO_2$ labeling experiment of grape berries : B, bag; C, cryostat (2-3°C); SG, gas syringe; V, valve; Ai-Fi (i=1-4), circuits (x6) of transparent bags (x4) containing grape bunches (one per bag) for six plants. (Reproduced with permission from reference (15). Copyright 2002 Elsevier.)*


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$^{13}CO_2$ was incorporated into the carotenoids as shown in Table I. Labeling of β-carotene (δ, ‰ : +109) was greater than that of xanthophylls (δ, ‰ : +62). At maturity there was a decrease in $^{13}C$-labelling of both carotenoids that could be explained by some carotenoid synthesis from veraison to maturity since $^{13}CO_2$ feeding ended before veraison. However the data indicate that a major part of carotenoids was synthesized before veraison since δ ‰ values are quite high at maturity. Furthermore in agreement with the literature, the concentration of carotenoids decreased significantly from veraison to maturity (*10*).

$^{13}CO_2$ was also incorporated into 3-oxo-α-ionol. The isotopic ratio of 3-oxo-α-ionol clearly increased from veraison to maturity suggesting that this compound was generated from the labeled carotenoid precursor during maturation. At maturity δ ‰ value of this compound was close to that of β-carotene and xanthophylls. All data points to the carotenoidic origin of 3-oxo-α-ionol.



**Table I. Isotopic Ratios and Concentrations of Carotenoids and 3-Oxo-α-ionol in Control and $^{13}CO_2$ fed Syrah Berries. (Reproduced from reference (*15*))**

| *Berries* | *β-Carotene* | *Xanthophylls* | *3-Oxo-α-ionol* |
|---|---|---|---|
| δ-values (δ, ‰) | | | |
| Labeled and sampled 26 July | +109 | +62 | +13.1, +22.7 |
| Labeled and sampled 19 September | +71 | +55 | +58.6, +61.1 |
| Control 19 September | -32 | -32 | -31, -31.5 |
| Concentrations mg $kg^{-1}$ | | | |
| Labeled and sampled 26 July | 5.6 | 5.6 | 29 |
| Labeled and sampled 19 September | 1.9 | 1.9 | 74 |

## Evidence of a Carotenoid Cleavage Dioxygenase (VvCCD1) from *Vitis vinifera*

The first carotenoid cleavage oxygenase (CCO) giving rise to volatile norisoprenoids was only reported in 2001 (*19*). Through the heterologous expression in *Escherichia coli* of the relevant CCO gene from *Arabidopsis thaliana* the formation of $C_{13}$-norisoprenoids from several carotenoids was demonstrated. The cleavage was occurring at 9,10 (9′,10′) double bonds of carotenoids. This paper has stimulated studies in several plant systems for the


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existence of CCOs which give rise to norisoprenoids. Since then CCOs have been identified in several plants and fruits such as *Vitis vinifera* (*9*), *Crocus sativa* (saffron) (*20*), *Petunia hybrida* (*21*), *Lycopersicon esculentum* (tomato) (*22*), *Cucumis melo* (melon) (*23*), *Chyrysanthenum morifolium Ramat* (*24*), *Citrus cv*. (*25*), *Fragaria ananassa* L. (strawberry) (*26*), *Zea mays* (*27*), *Coffea arabica* and *Coffea canephore* (*28*), *Rosa damascena* (*29*) *Osmanthus fragrans* Lour (*30*) and *Prunus persica* (*31*). This class of CCO cleaves carotenoids at the 9,10 (9′,10′) double bonds often with a large substrate specificity. They are classified as carotenoid cleavage dioxygenase 1 (CCD1). A dioxygenase mechanism has been proposed for CCDs since two oxygen atoms from $O_2$ are incorporated into the cleavage products instead of one oxygen atom from $O_2$ and one from water (case of monooxygenases from mammals) (*31*). Some CCDs are prone to cleave 9,10 bonds of apocarotenoids and as well as 5,6 (5′,6′) and 7,8 (7′,8′) double bonds of carotenoid precursors but further studies are needed to confirm this (*32*).

With regard to *Vitis vinifera* L. one clone from EST collection showed a full-length cDNA (VvCCD1) exhibiting homologies with CCD1 from *Arabidopsis thaliana* (*AtCCD1*) (*9*). A cytoplasmic location of VvCCD1 was proposed since no chloroplastic transit peptide could be predicted (*9*). This is in agreement with the proposition of cytosolic localization of CCD1 in plants and fruits in contrast to all other CCDs (CCD4, CCD7 and CCD 8) and NCED (Nine-*cis*-epoxycarotenoid dioxygenase) localized in the plastids (*32*). Cloning of VvCCD1 into β-carotene, zeaxanthin or lycopene accumulating *E.coli* strain resulted only in the discoloration of the colonies from *E.coli* accumulating zeaxanthin (*33*) (Figure 2).

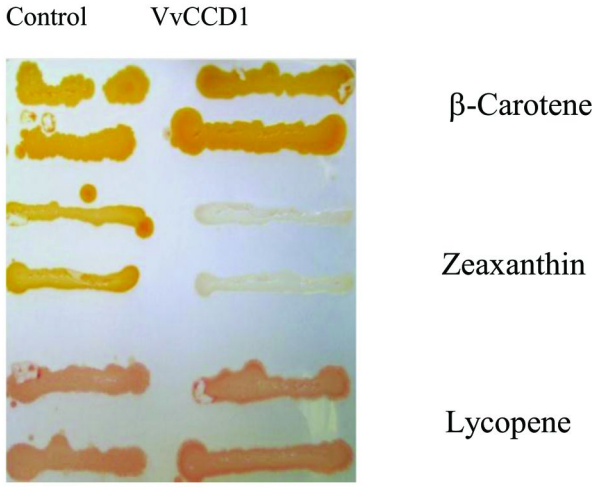


*Figure 2. Colonies from E.coli accumulating β-carotene, zeaxanthin, lycopene. VvCDD1 raw corresponds to the transformed E.coli with VvCCD1.*


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A simple spectrophotometric enzyme assay was developed to measure CCD activity (*34*). Incubation of zeaxanthin with recombinant VvCCD1 from *E.coli* allowed to identify 3-hydroxy-β-ionone and $C_{14}$-dialdehyde by GC/MS. HPLC analysis indicated that VvCCD1 cleaves this carotenoid at the 9,10 (9′,10′) positions as shown in Figure 3.

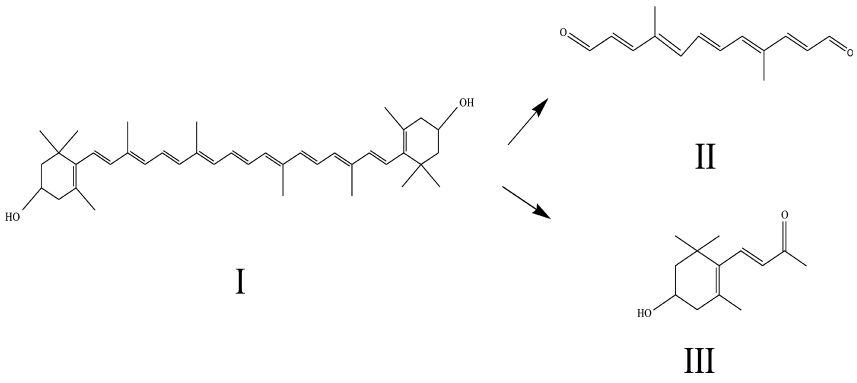


*Figure 3. Degradation products of zeaxanthin by VvCDD1. I: zeaxanthin; II: $C_{14}$-dialdehyde; III: 3-hydroxy-β-ionone.*

The same regiospecific cleavage by VvCCD1 occurred when lutein was used as substrate. Among the cleavage products 3-hydroxy-β-ionone and $C_{14}$-dialdehyde were detected (*34*). However neither β-carotene nor lycopene were accepted as substrates suggesting that carotenes are no substrate for this enzyme. The presence of β-ionone in grape and wine may then have several origins: (i) chemical oxidation of β-carotene (*35*), (ii) photooxygenation (*36*), (ii) co-oxidation mechanism under the action of lipoxygenase (*37*), and (iv) action of another grape CCD. In contrast to grape CCD1, recombinant CCD1 from *Arabidopsis thaliana* (*19*), from *Osmanthus fragrans* Lour. (*30*) were able to produce β-ionone from β-carotene. CCD1 from tomato was proposed to cleave β-carotene to yield β-ionone since silencing of the relevant CCD1 genes led to a significant decrease in the level of this flavor compound in ripe fruits (*22*). Similarly CCD1 from *Cucumis melo* was proposed to be involved in the formation of β-ionone from β-carotene through the studies involving up-regulation of the relevant gene (*23*).

## Expression of VvCCD1 During Grape Berry Development

Expression of VvCCD1 during grape berry ripening from two cultivars (Shiraz and Muscat) together with $C_{13}$-norisoprenoid levels have been studied (*9*). A significant induction of VvCCD1 expression (evidenced by real-time PCR) during the week prior to veraison was observed for both cultivars, with a two-fold induction for Muscat of Alexandria and a nearly four-fold one for Shiraz (Figure 4). Following veraison, the gene expression was almost stable


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during ripening. VvCCD1 expression level was higher in Muscat of Alexandria berries than in Shiraz. This could explain higher levels of $C_{13}$-norisoprenoids in the former. $C_{13}$-norisoprenoids were mainly detected in glyconjugated form and their level increased significantly after veraison. A strong increase was observed for the Muscat of Alexandria cultivar during the first week following veraison. Shiraz berries showed a progressive increase throughout ripening. Among eleven $C_{13}$-norisoprenoids detected, at maturity 4-oxo-β-ionol, 3-hydroxy-7,8-dihydro-β-ionone, 3-oxo-α-ionol, 3-hydroxy-β-ionone were the major ones. Only two primary cleavage products of carotenoids were detected : 3-hydroxy-β-ionone, a cleavage product of zeaxanthin or lutein, and 3-hydroxy-5,6-epoxy-β-ionone, a cleavage product of violaxanthin.



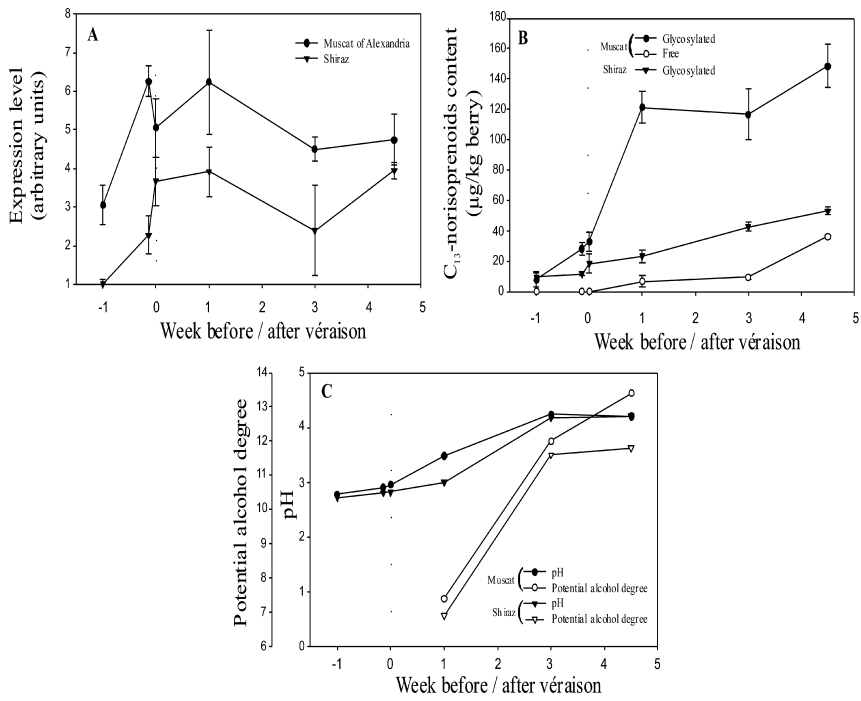


*Figure 4. Changes in the expression profile of VvCCD1 (A), in the levels of free and glycosylated $C_{13}$-norisoprenoids (B) and in pH and potential alcohol degree (C) in the berries from Muscat of Alexandria and Shiraz cultivars during grape berry development. Week 0 corresponded to veraison. (Reproduced with permission from reference (9). Copyright 2012 Oxford University Press.)*

## Secondary Transformations of $C_{13}$-Norisoprenoids

Several $C_{13}$-norisoprenoids with a large structural diversity have been identified in grape berries and wine leaves (1-4) suggesting that VvCCD1 cleavage products are prone to further transformations. A cell suspension culture

of cv. Gamay was studied for its ability to transform administrated β-ionone and dehydrovomifoliol (*38*). Both norisoprenoids were metabolized, leading in each case to several norisoprenoidic compounds in free and glycoconjugated forms. Structures of metabolites detected in grape cell cultures administrated with β-ionone are shown in Figure 5. The same metabolites were identified in the grape cell cultures administrated with dehydrovomifoliol except for 3-oxo-α-ionone and 3-hydroxy-7,8-dihydro-β-ionone. Administered compounds were reduced at the side chain or oxygenated mainly at carbons 3 or 4 of the cyclohexane ring. The transformations suggest the involvement of hydroxylases, oxidoreductases and glycosyltransferases. Interestingly, most of the metabolites from the administrated compounds are present in grape berries and wine leaves (*3*, *4*).



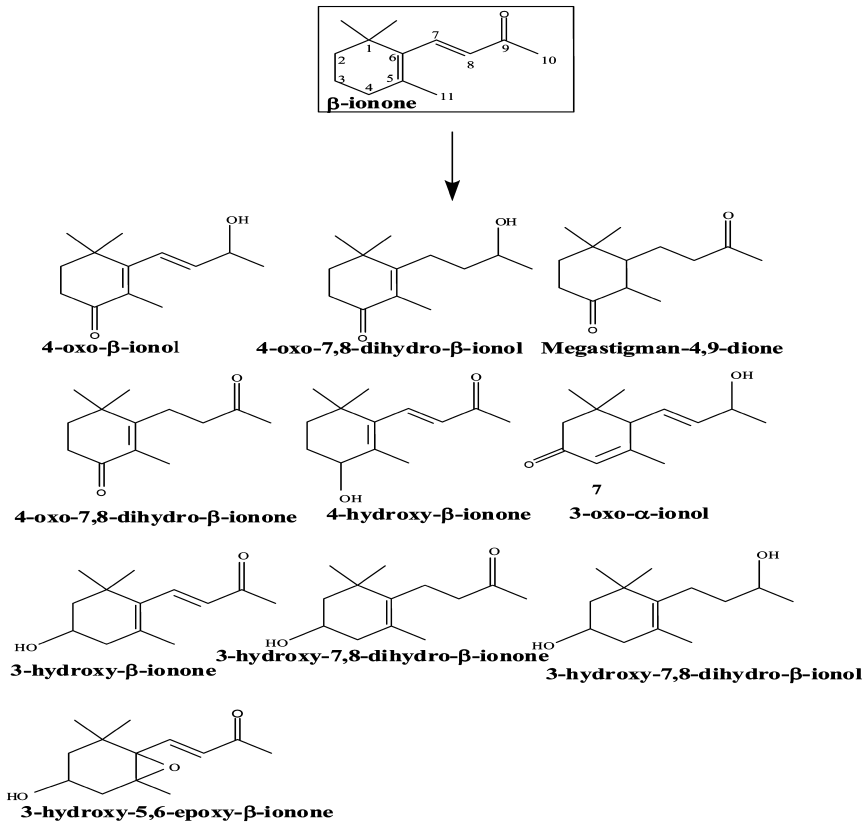


*Figure 5. $C_{13}$-norisoprenoids detected in grape cell cultures administrated with β-ionone. (Reproduced with permission from reference (38). Copyright 2012 Oxford University Press.)*

## Summary

A recombinant Carotenoid Cleavage Dioxygenase (CCD) from *Vitis vinifera* L. was able to symmetrically cleave zeaxanthin and lutein at the 9,10 (9′,10′) double bonds generating $C_{13}$-norisprenoids. On the contrary, β-carotene, amongst major carotenoids from grape was not a substrate. Expression of CCD gene was monitored by real-time PCR during berry development from Shiraz and Muscat of Alexandria cultivars. Gene expression was significantly induced approaching verasion. In parallel $C_{13}$-norisoprenoids level increased from verasion to maturity. $C_{13}$-norisoprenoids were prone to further modifications since a cell suspension culture from grape transformed two targeted $C_{13}$-norisoprenoids to several free and glycoconjugated compounds.



## Acknowledgments

The author of this chapter thanks his co-authors, who are identified in the references.

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