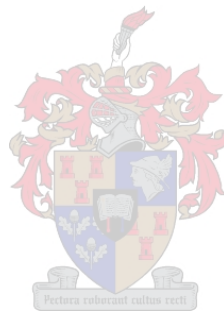


Carotenoid cleavage dioxygenases (CCDs) of grape

by

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Declaration

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Summary

Plant carotenoid cleavage dioxygenases (CCD) are a family of enzymes that catalyse the oxidative cleavage of carotenoids and/or apocarotenoids. Carotenoids are synthesised in plastids (primarily chloroplasts and chromoplasts), where they are involved in light-harvesting and protecting the photosynthetic apparatus from photo-oxidation. The carotenoid-derived apocarotenoids fulfil a number of roles in plants such as phytohormones, pollinator attractants and flavour and aroma compounds. Due to the floral and fruity characteristics that apocarotenoids contribute to wine, these C₁₃ compounds have received interest in grapevine (*Vitis vinifera* L.).

The CCD gene family in *Arabidopsis* consists of nine members, all encoding for enzymes that catalyse the cleavage of carotenoids. The enzymes in this family include 9-*cis*-epoxydioxygenases (NCEDs) and four classes of CCD. NCEDs and CCD7 and CCD8 are involved with plant hormone synthesis, e.g. abscisic acid (ABA) through cleavage by NCED and strigolactone (SL) through the sequential cleavage of carotenoids by CCD7 and CCD8, respectively. SLs are a fairly new class of plant hormone which are involved in several aspects of plant growth and development. The most extensively characterised role of SLs is their involvement in the inhibition of shoot-branching. CCD1 and CCD4 cleave a variety of carotenoids to form pigments and aroma compounds. For example, CCD1 forms β -ionone and β -damascenone, which are important varietal flavours of wine, and CCD4 is involved in synthesis of the pigment and aroma compounds of saffron and annatto.

CCD1 enzymes symmetrically cleave the 9,10 (9',10') double bonds of multiple carotenoids to produce a C₁₄ dialdehyde and two C₁₃ products. Additional CCD1 cleavage activity at 5,6 (5',6') double bonds of lycopene has been reported. Previous studies have shown that CCD1 isolated from *V. vinifera* (VvCCD1) was able to cleave multiple carotenoid substrates *in vitro*, namely zeaxanthin, lutein and β -carotene at 9,10 (9',10') double bonds and both the 5,6 (5',6') and 9,10 (9',10') double bonds of lycopene. None of the other VvCCDs, except VvCCD4a have been isolated (but no functionality was illustrated) and characterised yet. CCD4 enzymes also cleave carotenoids at the 9,10 (9',10') double bond positions. The presence of plastid-target peptides implies that the CCD4 enzymes have continuous access to carotenoids. Therefore it is suggested that CCD4s are responsible for carotenoid maintenance, where CCD1s contribute towards volatile production.

To test this hypothesis *VvCCD1*, *VvCCD4a* and *VvCCD4b* were isolated from *V. vinifera* (cv Pinotage) cDNA and cloned into a pTWIN1 protein expression vector. Substrate specificity of each VvCCD was tested by co-transforming a carotenoid accumulating *E. coli* strain with a CCD expression vector. Carotenoids synthesized by the bacteria were identified and quantified by UPLC-analysis, while the concentration of the apocarotenoids, were measured in the headspace of the bacterial cultures using HS-SPME-GC-MS. Several optimisations were done to minimize the natural degradation of the carotenoids; to ensure that the apocarotenoid formation is predominantly due to the enzymatic cleavage by the VvCCDs and not due to oxidation or other non-enzymatic degradation. The HS-SPME-GC-MS analysis indicated that all isoforms cleaved phytoene, lycopene and ϵ -carotene. Additionally *VvCCD1* cleaved a carotenoid involved in photosynthesis, namely β -carotene, while *VvCCD4a* cleaves neurosporene and *VvCCD4b* cleaves neurosporene and ζ -carotene, carotenoids not involved in photosynthesis.

This study has illustrated that *VvCCD1* cleave carotenoids necessary for photosynthesis and *VvCCD4s* cleave carotenoids which were not present in berry tissue, suggesting their role in carotenoid maintenance. Therefore *in planta* substrates for CCD1 could possibly be C_{27} apocarotenoids generated from enzymatic cleavage through CCD4 (role in carotenoid maintenance), CCD7 and/or photo-oxidation, which are then transported from the plastid to the cytosol or possibly C_{40} carotenoids that are released during senescence or when the plastid membrane is damaged, thus releasing important aroma compounds. Thus the identification of the *in vivo* substrates has contributed to the understanding the *in planta* functions of these enzymes.

Opsomming

Die plant ensiemfamilie van karotenoïedsplitsingdioksigenases (CCDs) kataliseer die oksidatiewe splitsing van karotenoïede en/of apokarotenoïede. Karotenoïede word in plastiede (primêr chloroplaste en chromoplaste) sintetiseer en is betrokke by lig-absorpsie en die beskerming van die fotosintetiese apparaat teen foto-oksidasie. Die apokarotenoïede afkomstig van karotenoïede dien onder meer as planthormone, geur- en aromakomponente en om bestuiwers aan te lok. Aangesien apokarotenoïede bydra tot die vrug- en blomgeure van wyn is die C₁₃-verbindings binne wingerd (*Vitis vinifera* L.) van belang.

Al nege lede van die CCD geenfamilie in *Arabidopsis* kodeer karotenoïedsplitsingsensieme. Die ensiemfamilie sluit 9-*sis*-epoksidioksigenases (NCEDs), en vier klasse CCD in. NCEDs en CCD7 en 8 is betrokke by die sintese van planthormone, naamlik absissiensuur (ABA) deur NCED en strigolaktone (SL) deur die opeenvolgende aksie van onderskeidelik CCD7 en CCD8. SL is redelik onlangs as planthormone indentifiseer en is betrokke by 'n verskeie aspekte van die groei en ontwikkeling van plante. Die rol van SL in inhibisie van vertakking is die beste gekarakteriseerde van hierdie aspekte. CCD1 en CCD4 splits 'n verskeidenheid karotenoïede om pigmente en aromakomponente te vorm. CCD1 vorm byvoorbeeld β-jonoon en β-damasenoon, beide belangrike kultivar-spesifieke wyngure. CCD4 vorm weer die pigment en aromakomponente van saffraan en annatto.

Die CCD1 ensieme splits die 9,10 (9',10') dubbelbindingsetels van verskeie karotenoïede simmetries en vorm een C₁₄-dialdehyd en twee C₁₃-produkte. Daar is voorheen melding gemaak van verdere splitsing deur CCD1 by die 5,6 (5',6') dubbelbindingsetels van likopeen. Vroeër is getoon dat die CCD1 isovorm wat uit *V. vinifera* geïsoleer is, naamlik VvCCD1, in vitro seaxantin, luteïen en β-karoteen by die 9,10 (9',10') dubbelbindingsetels kon splits, en likopeen by beide die 9,10 (9',10') en 5,6 (5',6') dubbelbindingsetels. Geen ander VvCCDs is al isoleer en funksioneel gekarakteriseer. VvCCD4a is isoleer, maar geen funksie is bepaal nie. CCD4 ensieme splits ook die 9,10 (9',10') dubbelbindingsetels van karotenoïede. Aangesien CCD4 ensieme 'n plastied-bestemmingspeptied besit behoort dié ensieme konstant toegang tot karotenoïede te hê, wat dui op hul rol in die handhawing van die karotenoïedbalans, terwyl CCD1-ensieme bydra tot die sintese van vlugtige verbindings.

Om hierdie hipotese te toets is VvCCD1, VvCCD4a en VvCCD4b uit *V. vinifera* (kv Pinotage) kDNS isoleer in binne 'n pTWIN1 proteïenuitdrukingsvektor kloneer. Die substraatspesifisiteit van elke VvCCD is getoets deur 'n karotenoïedakkumulerende *E. coil* stam te transvormeer met 'n CCD-uitdrukingsvektor. UPLC-analise is gebruik om karotenoïede wat deur die bakterium

sintetiseer is te kwantifiseer en identifiseer, terwyl die apokarotenoïedinhoud en -konsentrasie van die boruimte van die bakteriële kultuur met HS-SPME-GC-MS bepaal is. Verskeie aspekte van die proses is optimaliseer om natuurlike afbreking van karotenoïede te minimeer. Daardeur is verseker dat die apokarotenoïedvorming primêr vanweë die ensiematiese splitsing deur VvCCDs plaasvind en nie deur oksidasie of ander nie-ensiematiese afbreking. Die HS-SPME-GC-MS metings het aangedui dat al drie isovorme fitoëen, likopeen en ϵ -karoteen kan splits. VvCCD1 kan daarby β -karoteen splits, terwyl VvCCD4a neurosporeen, en VvCCD4b neurosporeen en ζ -karoteen kan splits, beide karotene wat nie betrokke is by fotosintese nie.

Dié studie toon dat VvCCD1 die karotenoïede splits wat benodig word vir fotosintese, terwyl beide VvCCD4 isovorme karotenoïede splits wat nie in druiwekorrels gevind word nie. Dit dui op hulle rol in die handhawing van karotenoïedpoele. Die *in planta* substrate vir CCD1 mag dus die C_{27} -apokarotenoïede wees wat deur CCD4 (as deel van karotenoïedhandhawing), CCD7 en/of foto-oksidasie gevorm word en na die sitosol vervoer word, of moontlik die C_{40} -karotenoïede wat tydens veroudering óf wanner die plastiedmembraan beskadig is in die sitosol vrygestel word. Die identifisering van die *in vivo* substrate het dus bygedra to die begrip van die *in planta* funksies van die ensieme.

This thesis is dedicated to my parents Tommy and Marlene and my sister Deirdre for all their love and support.

Biographical sketch

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Preface

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Chapter 1

**Introduction and
project aims**

1.1. Introduction

Plant carotenoid cleavage dioxygenases (CCD) are a family of enzymes that catalyse the oxidative cleavage of carotenoids and apocarotenoids. Plant carotenoid biosynthesis occurs in the plastid where carotenoids are primarily involved in photosynthesis (Demmig-Adams & Adams, 1996). Here they are bound in complexes with chlorophylls in the plastidial membrane. Carotenoids have different roles, depending on the type of plastid they are synthesised and present in. Carotenoids present in photosynthetically active chloroplasts are essential in protecting the photosynthetic apparatus from photo-oxidation and represent essential constituents of the light-harvesting and reaction centre complexes (Demmig-Adams & Adams, 1996; Pogson et al. 1998; Havaux & Niyogi, 1999; Park et al. 2002; Ahrazem et al. 2010). Those in chromoplasts are essential for attracting other organisms, such as seed-distributing herbivores and pollinating insects (Zhu et al. 2010), whereas carotenoids present in etioplasts and leucoplasts are essential in the formation of the phytohormones strigolactone (SL) (Gomez-Roldan et al. 2008; Umehara et al. 2008) and abscisic acid (ABA) (Cutler & Krochko, 1999; Zhang et al. 2009).

Both carotenoids and apocarotenoids are essential in the functioning of plants (reviewed by Auldridge et al. 2006a; Huang et al. 2009). As mentioned, their diverse functions are dependent upon their presence in a specific type of plastid or even within a specific location in the plastid (Gomez-Roldan et al. 2008; Umehara et al. 2008; Ahrazem et al. 2010; Zhu et al. 2010). The carotenoid-derived apocarotenoids fulfil a number of functions in plants such as hormones, pollinator attractants and flavour and aroma compounds. Several are commonly extracted and used as flavourants and colourants in the food and cosmetic industry (Huang et al. 2009). Well known examples include the orange-red colour of annatto, arising from the C₂₄ apocarotenoid bixin and the bright orange-red colour of saffron, which is mainly due to glycosides derived from the C₂₀ apocarotenoid crotenin (Bouvier et al. 2003). A number of apocarotenoids are present as volatile aroma compounds which are important in many fruits and flowers (reviewed by Kloer & Schulz, 2006) or which contribute to the varietal flavour and aroma of grapes and wine (Baumes et al. 2002; Mendes-Pinto, 2009).

The first protein found to specifically cleave carotenoids, viviparous14 (VP14), was identified by the analysis of viviparous abscisic acid-deficient mutant maize (Schwartz et al. 1997; Tan et al. 1997). The pioneering work done on the VP14 facilitated the discovery of related enzymes in different plant species and other organisms (Tan et al. 2003). The CCD gene family in *Arabidopsis thaliana* consists of nine members, all encoding for enzymes that catalyse the

cleavage of carotenoids. Members of the family include the *9-cis-epoxydioxygenases* (*NCEDs*), that forms ABA (Schwartz et al. 1997); *CCD1s* that code for enzymes that cleave a broad range of carotenoids forming volatile aroma compounds (Simkin et al. 2004; Auldridge et al. 2006b); *CCD4s* encode enzymes catalysing the cleavage of carotenoids forming aroma and pigment compounds (Bouvier et al. 2003; Ohmiya et al. 2006; Huang et al. 2009) and *CCD7s* and *CCD8s* that encode enzymes that catalyse the sequential cleavage of carotenoids to form SL, the hormone involved in the inhibition of shoot branching (Auldridge et al. 2006b; Domagalska & Leyser, 2011; Waters et al. 2012). The broad range of apocarotenoids produced by CCD cleavage and the diverse biological roles of these compounds demonstrate the importance of CCDs to plants in functions as diverse as drought tolerance, attractors of pollinators, as well as growth and developmental regulation (Bouvier et al. 2003; reviewed by Bouvier et al. 2005).

Five members of the *Arabidopsis NCED* family have been implicated in ABA biosynthesis, a hormone that plays an important role in the closing of stomata and drought tolerance, seed development and dormancy and sugar sensing (Schwartz et al. 1997). In higher plants, ABA is derived from C_{40} -*cis*-epoxycarotenoids, either 9'-*cis*-neoxanthin or 9'-*cis*-violaxanthin or both, which are cleaved by the NCED at the 11,12 (11',12') double bond to produce xanthoxin, the direct C_{15} precursor of ABA (Cutler & Krochko, 1999, Zhang et al. 2009).

CCD1s are known to catabolise a wide variety of all-*trans*- and 9-*cis*-carotenoids as well as epoxycarotenoids. CCD1s symmetrically cleave 9,10 (9',10') double bonds of multiple carotenoid substrates to produce two C_{13} products and a C_{14} aldehyde. An additional cleavage activity for CCD1 has been reported at the 5,6 (5',6') double bonds of lycopene (Hung et al. 2009). OsCCD1 from rice can cleave lycopene at the 7,8 (7',8') double bonds (Ilg et al. 2009). CCD1 contributes to the formation of important apocarotenoid volatiles (β -ionone, β -cyclocitral, geranylacetone and pseudoionone) in the fruit and flowers of several plant species (Schwartz et al. 2001; Simkin et al. 2004; Mathieu et al. 2005; Vogel et al. 2008; García-Limones et al. 2008; Huang et al. 2009). Next to NCEDs, is CCD1 the best studied enzyme of this family due to its involvement in C_{13} apocarotenoid-based flower scent, as well as fruit and wine biosynthesis (Baumes et al. 2002; Mendes-Pinto, 2009).

In planta, CCD1 and CCD4 differ in their subcellular location; CCD1 is cytosolic or associated with the outer membrane of the chloroplast, whereas CCD4 is plastidic. The presence of plastid target peptides and the confirmed plastid localisation of the CCD4 enzymes allow these enzymes direct access to carotenoid substrates, signifying a likely role in carotenoid maintenance and apocarotenoid synthesis (Rubio et al. 2008; Brandi et al. 2011). Plants generally produce two

CCD4 isoforms (isozymes), CCD4a and CCD4b, that may act on different substrates and thus have different biological functions in plants (Ohmiya et al. 2006; Huang et al. 2009). The current data suggests that due to their different subcellular localisation, CCD1s only contribute towards volatile production, whereas CCD4s possibly control carotenoid degradation (Brandi et al. 2011). Although a large amount of work has been done investigating CCD enzymes *in vitro*, the *in planta* function(s) of CCDs are relatively unexplored. Huang et al. (2009) heterologously expressed five CCD4 genes that were isolated from different plant sources. The enzymatic assays revealed that the recombinant proteins derived from the different CCD4 genes oxidatively cleaved the substrates at the same positions (9,10 and 9',10' double bonds) and that the biological and biochemical functions may differ as the expressions patterns vary and they accept different substrates. Despite their importance in plant biology and physiology, functional analysis studies, as well as substrate specificity studies of CCDs are still not exhaustive, since the typical *in vitro* assays used for their analyses present some problems, mainly since carotenoid substrates are instable and can also be degraded non-enzymatically to form the same products as an enzymatic cleavage.

CCD7 and CCD8 were first characterised in *A. thaliana*, as the remaining members of the NCED/CCD family. *In vitro* AtCCD7 cleaved β -carotene at the 9,10 (9',10') double bond position, generating the C₂₇ compound β -apo-10'-carotenal and the C₁₃ compound β -ionone. When AtCCD7 was co-expressed in *E. coli* with AtCCD8, β -apo-13-carotenal was additionally identified, which was not present when AtCCD8 was expressed on its own. The β -apo-13-carotenal was formed by a secondary cleavage of the β -apo-10'-carotenal (formed by AtCCD7), at the 13,14 (13',14') double bond position (Schwartz et al. 2004; Walter et al. 2010). CCD7 and CCD8 have subsequently been linked to the production of SL; the encoding genes, the pathway they are involved in and SL functions in plants are actively researched, since limited information is currently available in non-model plants on them. This is also true for the CCDs in general and particular attention is currently given to study CCDs from crop plants.

One such non-model and important crop plant that would benefit from a more detailed analysis of the CCD encoding gene family is grapevine. The cultivars of the European grape, *Vitis vinifera* L., form the basis of the international wine industry, as well as the table grape and raisin industries of the world and display enormous variability in flavour and aroma composition. For the wine industry, in particular, the berry aromatic potential is an important quality impact factor, since the berry metabolome is the matrix for the wine fermentation. Carotenoid-derived aroma compounds are known to be the source of important varietal flavours e.g. β -ionone and β -damascenone (Winterhalter & Schreier, 1994; Winterhalter & Rouseff, 2002; Baumes et al.

2002; Mendes-Pinto, 2009). Moreover, a clear inverse correlation was found in wines, showing an increase in volatile compounds as carotenoids levels drop (Razungles et al. 1993). Carotenoid metabolism and the functions of carotenoids and their derived products are considered important aspects to study in grapevine due to the role of carotenoids in photosynthesis, photo-protection and aroma-precursor production (Young et al. 2012). The study of CCDs in these processes is crucial and this family of enzymes is still relatively unexplored in grapevine.

1.2. Project aims

The aim of this study was the *in vitro* functional characterisation of the carotenoid cleavage dioxygenase (CCD) gene family in grapevine (*V. vinifera*). This includes the identification and isolation of putative VvCCDs, the determination of the expression levels and patterns of the VvCCDs in different plant organs and different stages of berry development and the determination of the substrate range for the VvCCDs. Previous studies have reported the isolation of VvCCD1 and illustrated that this enzyme was able to cleave multiple carotenoid substrates *in vitro*. VvCCD1 cleaved zeaxanthin and lutein to produce 3-hydroxy- β -ionone, but could not cleave β -carotene according to Mathieu et al. (2005, 2007). Lashbrooke (2010) subsequently showed that VvCCD1 could cleave lycopene (producing 6-methyl-5-hepten-2-one (MHO) and pseudoionone) and β -carotene (producing β -ionone), in addition to the previously identified substrates. To date no other VvCCDs, except VvCCD4a, have been isolated and/or characterised, although Guillaumie et al. (2011) identified and isolated a putative VvCCD4a; they were unable to demonstrate functionality. The motivation for the current study is mainly linked to the fact that the identification of the *in vivo* substrates will help in understanding the *in planta* functions of these enzymes. Validating the VvCCD enzyme functions, clarifying their substrates and investigating the expression patterns of the encoding genes will shed light on their roles in plants. The following specific aims have been formulated for this study:

- i) *In silico* screening of the grapevine genome for putative VvCCD-encoding genes;
- ii) Expression analysis of the identified VvCCDs, as well as VvCCD1, in different *V. vinifera* organs and stages of berry development;
- iii) Isolation of putative VvCCD4, VvCCD7 and VvCCD8-encoding genes;
- iv) The *in vivo* characterisation of the substrate specificity of the isolated VvCCDs, as well as VvCCD1, through the transformation of carotenoid-accumulating *E. coli* strains with relevant expression cassettes, determination of substrates present via UPLC analysis and subsequent measurement of the apocarotenoids formed via GC-MS analysis.

The results obtained are presented in Chapter 3 of this thesis, after a concise literature review on plant CCDs in Chapter 2. The main findings and their impact are highlighted and concluded on in Chapter 4.

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Chapter 2

Literature review

The role of carotenoid cleavage dioxygenases (CCDs) in
plants

2.1. Introduction

2.1.1. Carotenoids

Carotenoids are isoprenoid pigments that are present in the membranes of all phototrophic as well as many heterotrophic organisms where they serve a large number of functions (Goodwin, 1980). Carotenoids first emerged in primitive organisms, and due to their inflexible conjugated double-bond backbones most likely evolved as lipid molecules to strengthen membranes (reviewed by Walter & Strack, 2011). Carotenoids are derived from the linear tetraterpene phytoene (C₄₀), containing up to 11 conjugated double bonds. The nature and number of these double bonds determines the emission maxima and excitation wavelengths and consequently the spectral properties of these pigments (Ritz et al. 2000). In plants their presence is revealed by the rich colours of fruits, flowers and autumn leaves in the yellow to red spectrum. Carotenoids are synthesized in plastids (primarily the chloroplasts and chromoplasts) and have different roles, depending on which type of plastid they are present in. In chloroplasts, carotenoids are involved in a number of functions vital for photosynthesis such as light-harvesting, the reaction centre complexes and protecting the photosynthetic apparatus from photo-oxidation (Ahrazem et al. 2010; Zhu et al. 2010). Carotenoids thereby facilitate non-photochemical quenching and photomorphogenesis and the prevention of lipid peroxidation (Demmig-Adams & Adams, 1996; Pogson et al. 1998; Havaux & Niyogi, 1999; Park et al. 2002; Ahrazem et al. 2010). The primary function of carotenoids in chromoplasts appears to be for attracting other organism, such as herbivores for seed distribution and insects for pollination (Zhu et al. 2010). Carotenoids present in etioplasts and leucoplasts are essential in the formation of the phytohormone strigolactone (SL) (Gomez-Roldan et al. 2008; Umehara et al. 2008).

2.1.2. Apocarotenoids

Apocarotenoids are a class of terpenoid compounds generated by oxidative cleavage of carotenoids. The assortment of apocarotenoids results from the large number of carotenoid precursors (more than 700 have been identified), variations in specific cleavage site and modification(s) after cleavage (Schwartz et al. 2001). In plants and cyanobacteria, apocarotenoids are largely found in the thylakoid membrane, where they act as photoprotective and accessory pigments (Markwell et al. 1992). Dependent upon the size of the chromopore, apocarotenoids can absorb visible light and are therefore useful as colour pigments for attracting pollinators and seed dispersal agents. Well known examples are the orange-red colour of annatto, arising from the C₂₄ apocarotenoid bixin (Figure 1) and the bright orange-red colour of saffron (Figure 1), which is mainly due to glycosides derived from the C₂₀ apocarotenoid crocetin (Figure 1) (Bouvier et al. 2003). A number of apocarotenoids are present as volatile aroma

compounds; for example the characteristic component of rose scent is the C₁₃ volatiles β-ionone and β-damascenone (Huang et al. 2009a). Apocarotenoid volatiles that are emitted by many flowers or vegetative tissues favour plant-insect interactions (Donaldson et al. 1990; McQuate & Peck, 2001; Azuma et al. 2002). They are also the key components of the aroma produced during the development of some fruit and tobacco curing (Winterhalter & Rouseff, 2002; Camara & Bouvier, 2004). Due to the floral and fruity characteristics that apocarotenoids contribute to wine, these C₁₃ norisoprenoids have received interest in grapevine (*Vitis vinifera* L.) berries (Baumes et al. 2002; Mendes-Pinto, 2009).

Several examples have been described where apocarotenoids act as repellents, chemo-attractants, growth simulators and inhibitors (Bouvier et al. 2003; reviewed by Bouvier et al. 2005). Certain apocarotenoids are hormones involved in the regulation of plant architecture and growth. The best example is abscisic acid (ABA) (Figure 1), which plays an important role, amongst others, in the regulation of seed development, drought resistance and sugar sensing (Schwartz et al. 2003; Taylor et al. 2005). It is derived from the C₁₅ apocarotenoid xanthoxin. The biosynthesis of ABA is mainly regulated at the initial carotenoid cleavage step leading to xanthoxin (Schwartz et al. 2003). The existence of the additional apocarotenoid phytohormone, namely SL (Figure 1), has arisen over the past few years from a series of mutants exhibiting an increased shoot branching phenotype (Koltai & Kapulnik, 2011; reviewed by Walter & Strack, 2011).

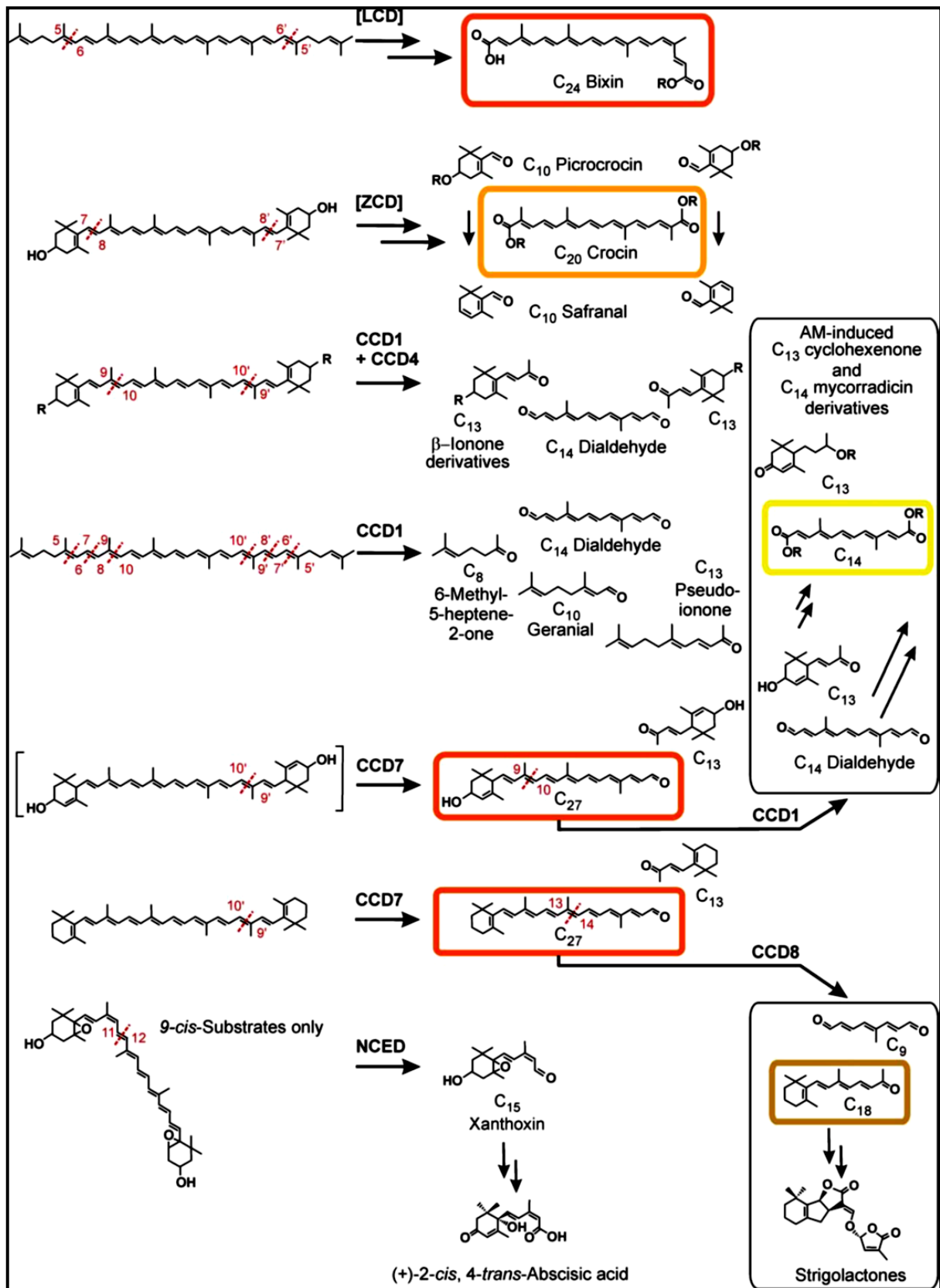


Figure 1. The specific enzymatic cleavage reactions of carotenoids or apocarotenoids catalysed by various CCDs from plants. Cleavage sites in substrates are indicated by dotted red lines. Known chromophores in cleavage products are boxed in colour. Two cases of sequential cleavage are highlighted and their end products are boxed in black. Square brackets indicate predicted structures or limited characterization (figure from Walter & Strack, 2011).

2.2. Carotenoid metabolism

Carotenoid biosynthesis occurs in plastids of plant cells. Carotenoids can be degraded by chemical; photochemical, oxidase-coupled mechanisms, or enzymatically (Mathieu et al. 2005). Carotenoids are tetraterpenoids; i.e. they are comprised of eight condensed C₅ isoprenoid precursors that generate a linear C₄₀ backbone (Britton, 1983). They are derived from the plastid-localised 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway for which pyruvate and glyceraldehyde-3-phosphate act as the initial substrates leading to the synthesis of geranylgeranyl diphosphate (GGPP) (Figure 2). The condensation of two GGPPs by phytoene synthase (PSY) forms 15-*cis*-phytoene, which represents the first step in the carotenoid biosynthetic pathway (Cazzonelli & Pogson, 2010).

The production of the all-*trans*-lycopene from 15-*cis*-phytoene in plants requires a series of four reactions, which are carried out sequentially by phytoene desaturase (PDS), ζ -carotene isomerase (Z-ISO), ζ -carotene desaturase (ZDS) and occasionally carotenoid isomerase (CRTISO) (Figure 2). The product which is generated from the first desaturation is 9,15,9'-tri-*cis*- ζ -carotene, which is non-enzymatically isomerised by light (photo-isomerised) or in the absence of light enzymatically by Z-ISO to yield 9,9'-di-*cis*- ζ -carotene, which is the substrate of ZDS. The end product of the desaturation reactions is non-enzymatically converted to all-*trans*-lycopene by light and chlorophyll in green tissue. In the dark and in non-photosynthetic tissue, the carotenoid isomerase (CRTISO) is required (Breitenbach & Sandmann, 2005).

Carotenoid biosynthesis branches after lycopene to produce ϵ - and β -carotene by the introduction of hydroxyl moieties into the cyclic end groups by carotene ϵ -hydroxylase and/or β -carotene hydroxylase, which results in the formation of lutein (from α -carotene) and zeaxanthin (from β -carotene). This divergence is regulated by the enzymes ϵ -lycopene cyclase (ϵ -LCY) and β -lycopene cyclase (β -LCY). These enzymes determine the oscillation between the most abundant carotenoids and xanthophylls, specifically the relative amounts of lutein (β,ϵ rings) and β -carotene (β,β rings) (Tian et al. 2004; Galpaz et al. 2006; Kim & DellaPenna, 2006; reviewed by Farré et al. 2010; reviewed by Walter & Strack, 2011). The ϵ,ϵ -ring formation of ϵ -carotene is rare and has only been identified in a few species (e.g. lactucaxanthin in the genus *Lactuca*) (Phillip & Young, 1995).

One of the main functions of zeaxanthin is the role in the xanthophyll or violaxanthin cycle. This occurs in the thylakoid membranes of higher plants to offer protection to the photosynthetic apparatus against high irradiance via the dissipation of excessive energy into heat. Several xanthophylls and co-factors participate in this cycle. Zeaxanthin can be converted to antheraxanthin and then to violaxanthin via ZEP, which catalyses two of the epoxidation

reactions (Marin et al. 1996; reviewed by Walter & Strack, 2011). Violaxanthin is then converted by neoxanthin synthase (NXS) to neoxanthin (North et al. 2007; reviewed by Farré et al 2010). Some plants also employ a lutein epoxide cycle. Different lutein or zeaxanthin epoxidation kinetics by zeaxanthin epoxidase (ZEP) might allow for a combination of slow and rapid reversible modulation of photoprotection and light harvesting (Garcia-Plazaola et al. 2007; Esteban et al. 2009). The C_{40} 9-*cis*-epoxycarotenoid precursors are cleaved by 9-*cis*-epoxycarotenoid dioxygenase (NCED) to xanthoxin and this is followed by the two step conversion by ABA aldehyde to ABA (Schwartz et al. 2003).

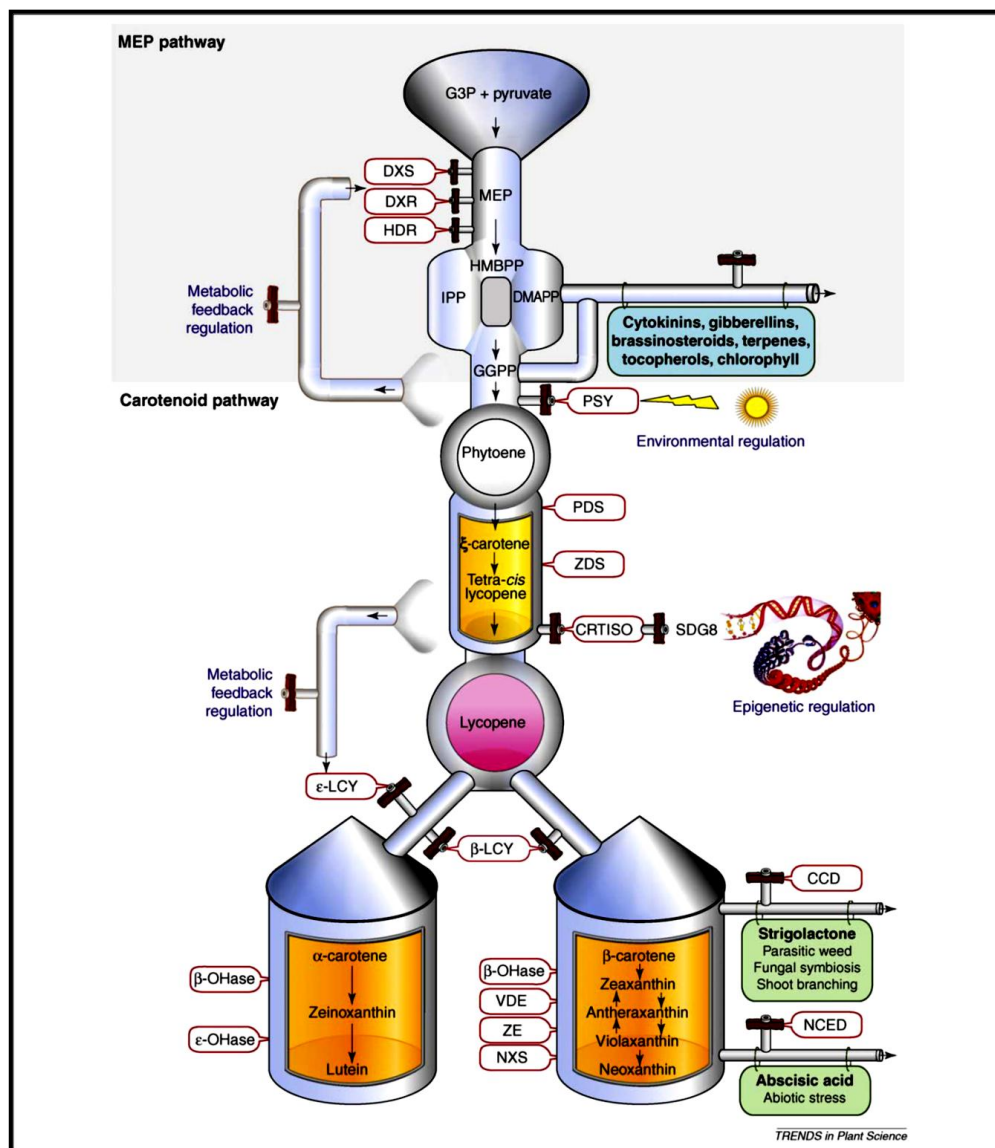


Figure 2. This figure illustrates the major reactions in higher plant carotenoid biosynthetic pathway. The enzymes, carotenoids and their precursors (pipes), carotenoid sinks (barrels), carotenoid-derived signalling hormones (green signals) and the MEP isoprenoid-derived metabolites (blue sign) are illustrated. Abbreviations: β-LCY, β-cyclase; β-OHase, β-hydroxylase; CCD, carotenoid cleavage dioxygenase; CRTISO, carotenoid isomerase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxyxylulose-5-phosphate synthase; ε-LCY, ε-cyclase; ε-OHase, ε-hydroxylase; GGPP, geranylgeranyl diphosphate; HDR, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NXS, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; SDG8, histone methyltransferase; VDE, violaxanthin de-epoxidase; ZDS, ζ-carotene desaturase; and ZE, zeaxanthin epoxidase (figure from Cazzonelli & Pogson, 2010).

2.3. The Carotenoid Cleavage Dioxygenase (CCD) family: History and categorisation

The first protein found to specifically cleave carotenoids, viviparous14 (VP14), was identified by the analysis of viviparous ABA-deficient mutant maize (Schwartz et al. 1997; Tan et al. 1997). Analysis of the enzymatic activity of VP14 showed that it cleaves 11,12 (11',12') double bonds of the 9-*cis* isomers of violaxanthin and neoxanthin to yield xanthoxin (C₁₅ apocarotenoid), which is the precursor of ABA. The pioneering work done on the VP14 facilitated the discovery of related enzymes in different plant species and other organisms (Tan et al. 2003). Based on their substrate specificity, VP14 and its orthologues have been named 9-*cis* epoxy-carotenoid cleavage dioxygenases (NCEDs) (Auldrige et al. 2006a).

Two types of carotenoid dioxygenases have been identified in plants: 9-*cis* carotenoid cleavage dioxygenases (NCEDs) and carotenoid cleavage dioxygenases (CCDs). In *Arabidopsis thaliana*, the CCD family has nine members: the five *NCEDs* (*NCED2*, *NCED3*, *NCED5*, *NCED6* and *NCED9*) are involved in the biosynthesis of the plant hormone ABA (Figure 3) and the four *CCDs* (*CCD1*, *CCD4*, *CCD7* and *CCD8*) are involved in various carotenoid cleavage reactions. The *CCDs* (*CCD1*, *CCD4*, *CCD7* and *CCD8*) have low sequence homology to the *NCEDs* and the substrate specificity and enzyme activity of the encoded products also differs from those of the *NCEDs* (Tan et al. 2003; reviewed by Ohmiya, 2009). The majority of the NCEDs/CCDs have been shown to reside in plastids, where their substrates are also localised. The only exception is CCD1 which acts in the cytosol, or in association with the outer membrane of the plastid (Vidi et al. 2006; Ytterberg et al. 2006; reviewed by Floss & Walter, 2009).

2.4. Enzymatic cleavage of carotenoids by CCDs: Mode of action

The crystal structure of plant CCDs has not been resolved, but the three-dimensional structure of a related family member, apocarotenoid 15,15'-oxygenase (ACO) from the cyanobacterium *Synechocystis* was identified by Kloer et al. (2005). A high degree of similarity exists between the amino acid sequences of ACOs and plant CCDs at important structural regions of the proteins (reviewed by Kloer & Schulz, 2006). *In vitro*, ACO cleaves the 15,15' double bond and its preferred substrates are apocarotenols of C₂₇-C₃₀ chain length with hydroxylated ionone rings. ACOs have a proposed seven-bladed β-propeller tertiary structure. The four characteristic histidine side chains holding the catalytic ferrous iron reside in the propeller axis (Figure 3). A hydrophobic patch (Figure 3) on the surface of ACO sits at the entrance of the active site tunnel and is proposed to be important for regulation and substrate channelling or availability (reviewed by Auldrige et al. 2006b). The two ACO monomers (present within the crystal forms) associate at this site to form a combined hydrophobic patch, which might be utilised for membrane localisation and extraction of non-polar substrates (Kloer et al. 2005).

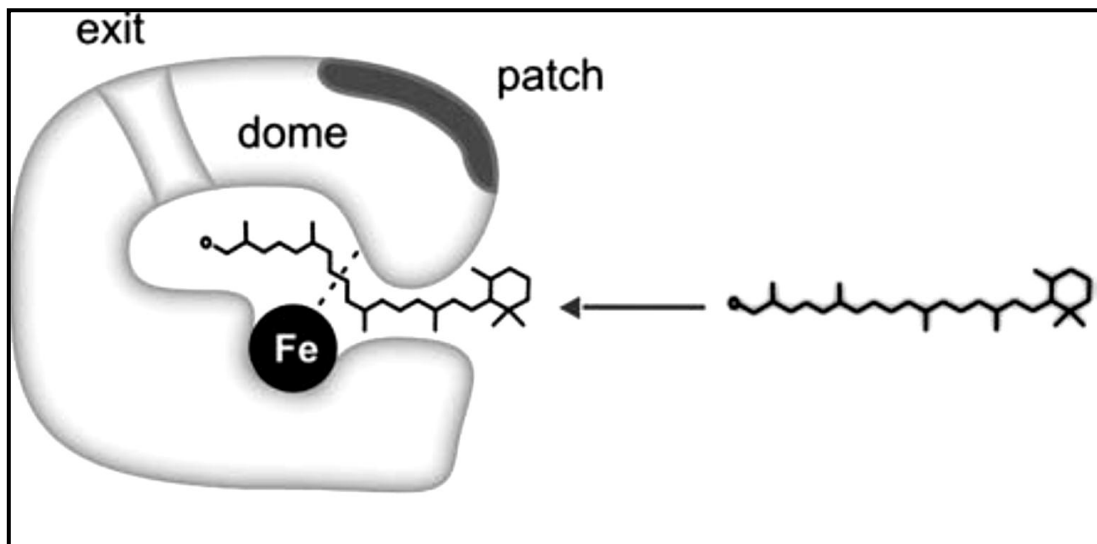


Figure 3 This figure depicts a model for CCD/ACO structure. The tunnel entrance, which determines the substrates that the enzyme accepts, the active site, which contains an Fe^{2+} ion essential for cleavage, and the non-polar patch, allowing binding to non-polar membranes is illustrated (figure from Kloer & Schulz, 2006).

The mechanism for ABA biosynthesis by the dioxygenase cleavage of the *9-cis*-carotenoid bond has been proposed (Shwartz et al. 2003), but the structure and nature of the determinants of specificity of the dioxygenase remains unknown (Messing et al. 2010). Messing et al. (2010) used the structure of VP14 to identify amino acid residues that played a role in determining which bond was cleaved and the position of the substrate. These residues were contrasted and compared to the plant carotenoid cleavage dioxygenase family. As was suggested by Kloer & Schultz, (2006), the β -propeller portion of the structure was a conserved characteristic throughout the CCD enzyme family, as it was present in both the prokaryotic apocarotenoid 15,15'-oxygenase (ACO) and the eukaryotic VP14. The helical domain may be the structural feature that differentiates plant CCDs from the rest of the members of the CCD family (Messing et al. 2010). Messing et al. (2010) identified the noteworthy level of sequence identity between VP14, NCEDs and the CCDs family in plants. This made VP14 a suitable/useful prototype to use in the investigation of these enzymes. The coordinates of the VP14 substrate model was used as the template to construct a homology model of *Zea mays* CCD1 (ZmCCD1). Comparisons between the two models identified that the differences in substrate specificity were due to three crucial regions in the structures. Mutational studies done on ZmCCD1, validated the use of VP14 as a template for mapping the important residues in the substrate tunnels of plant CCDs and provided a basis for understanding their substrate specificity (Messing et al. 2010).

2.5. Non-enzymatic degradation of carotenoids

Carotenoid degradation might not be limited to enzymatic degradation, but could be due to the damage to (or destruction of) C₄₀ carotenoids by photochemical processes or other conditions of oxidative stress. Carotenoids, among other antioxidants present in chloroplasts, are considered to be the first line of defence of plants against O₂ toxicity (Cogdell & Frank, 1987; reviewed in Edge et al. 1997; Triantaphylidès & Havaux, 2009) and are therefore the products derived from their oxidation, are potential candidates for protection (Ramel et al. 2012). Enzymatic cleavage or non-enzymatic cleavage of a specific carotenoid can yield the same or different products. Ramel et al. (2012) demonstrated that carotenoid oxidation products accumulated in light-stressed conditions of *Arabidopsis* plants. The authors speculated that the involvement of enzymes (carotenoid cleavage dioxygenases) in the production of these products was unlikely.

2.6. The role of CCDs in plants

This section contains a summary of what is known of the functional roles of CCDs in plants in general, concluding with a brief summary of the current state of the art for grapevine.

2.6.1. The 9-cis-epoxycarotenoid dioxygenase (NCED) sub-family and their role in ABA biosynthesis

The best studied plant apocarotenoid is the phytohormone ABA. One of the classes of enzymes involved in plant ABA biosynthesis is called the NCEDs. NCEDs are plastidic, and are therefore co-localised with carotenoids (Tan et al. 2003; reviewed by Floss & Walter, 2009). NCEDs are unique among other carotenoid cleavage oxygenases (CCOs) in that they accept only *cis*-isomers of their substrates (Figure 4) (Tan et al. 2003; reviewed by Walter & Strack, 2011). ABA levels rise under stress conditions as well as during seed and bud dehydration (Cutler & Krochko, 1999; Ren et al. 2007; Endo et al. 2008). The cleavage reaction of 9-cis-violaxanthin and 9-cis-neoxanthin is the rate-limiting step in the biosynthesis of ABA (Cutler & Krochko, 1999). Mutations of *NCED* genes in maize (*Zea mays*) resulted in droopy phenotypes and reduced ABA levels/concentrations (Tan et al. 1997). ABA is also considered important for grapevine berry ripening (Wheeler et al. 2009).

NCED-encoding genes have been isolated from numerous species including maize (*Zea mays*) (Tan et al. 1997), tomato (*Solanum lycopersicum*) (Burbidge et al. 1999), bean (*Phaseolus vulgaris*) (Qin & Zeevaart, 1999) avocado (*Persea americana*) (Chernys & Zeevaart, 2000), cowpea (*Vigna unguiculata*) (Iuchi et al. 2000), *Arabidopsis thaliana* (Iuchi et al. 2001) potato (*Solanum tuberosum*) (Destefano-Beltrán et al. 2006), orange (*Citrus sinensis*) (Rodrigo et al. 2006) and grape (*V. vinifera*) (Zhang et al. 2009). In some plant species, *NCED*-like genes were

identified, comprising of a small multi-gene family, with only a subgroup involved in the stress responses and regulation of ABA biosynthesis (Chernys & Zeevaart, 2000; Tan et al. 2003; Rodrigo et al. 2006; Qin et al. 2008). Five members of the *Arabidopsis* NCED family were identified, namely AtNCED2, AtNCED3, AtNCED5, AtNCED6 and AtNCED9. All five AtNCEDs are targeted to the plastid, although they differ in their binding activity to the thylakoid membrane (Tan et al. 2001). AtNCED2, AtNCED3 and AtNCED6 are found in both the stroma and thylakoid membrane-bound compartments. AtNCED5 is bound to thylakoids and AtNCED9 is soluble in the stroma (Tan et al. 2003). Expression analysis of the *NCEDs* in *avo* (Chernys & Zeevaart, 2000); tomato (Thompson et al. 2000); *Arabidopsis* (Tan et al. 2003) and in other higher plants (Han et al. 2004) has suggested a key role of the NCED proteins in response of vegetative tissues to water stress and significant associations between expression and ABA accumulation were reported.

Transgenic *Arabidopsis* plants overexpressing *AtNCED3* displayed enhanced water stress resistance and increased ABA levels (Iuchi et al. 2001). These results were also found in tobacco overexpressing *LeNCED1* (Tung et al. 2008) and *PvNCED1* (Qin & Zeevaart, 2002), in *Arabidopsis* plants by an ectopic expression of a peanut *NCED* (Wan & Li, 2006) and in transgenic maize plants (Parent et al. 2009). *NCED* knock-outs showed weakened ability for ABA biosynthesis in stressed leaves (Burbidge et al. 1999; Iuchi et al. 2001). Similar results were obtained with carotenoid-deficient mutants (Kang & Zuber, 1989; Du et al. 2010).

Zhang et al. (2009) analysed the expression of *NCED1* isolated from peach fruit (*PpNCED1*) and *V. vinifera* (*VvNCED1*) during development and ripening. The genes were expressed only at the initial stages of ripening in both peach and grape, when the ABA was highest. Therefore the expression of these genes initiated ABA biosynthesis at the onset of ripening, clearly indicating that *NCEDs* are transcriptionally regulated. Sun et al. (2010) demonstrated a correlation between *VvNCED1* and ABA levels in peel, seed and pulp. A clear developmental pattern of expression of *VvNCEDs* exists in berries and the various isozymes have different expression patterns as illustrated in Young et al. (2012). Young et al. (2012) illustrated that *VvNCED2* expression was down-regulated during berry development and *VvNCED1* (*VvNCED3* in the study) expression peaked at the véraison stage of berry ripening. The expression of the *VvNCED* genes correlates with the levels of ABA present (Sun et al. 2010).

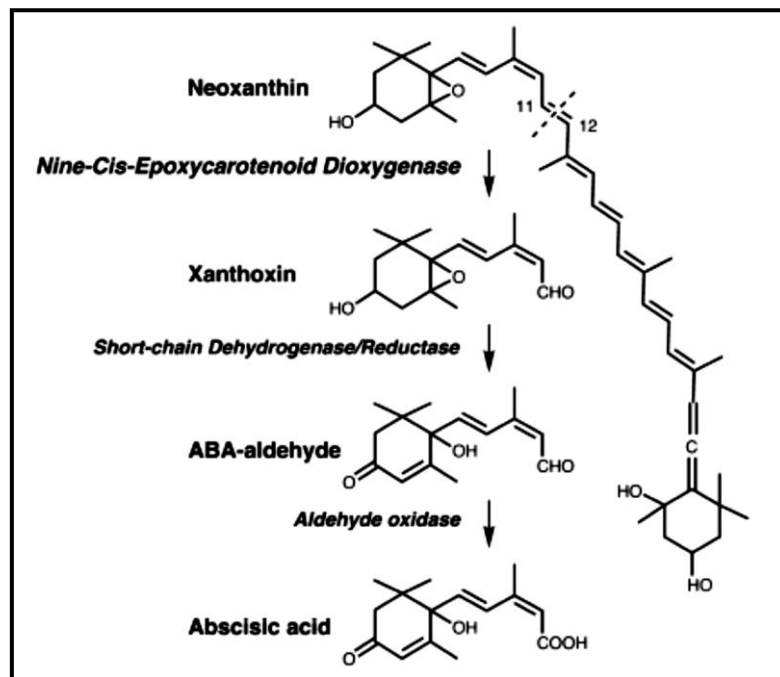


Figure 4. This figure illustrates the biosynthetic pathway of Abscisic acid as (figure from Camara & Bouvier, 2004).

2.6.2. Carotenoid cleavage dioxygenase (CCD) sub-family and their roles in plants

CCDs have low sequence homology to the NCEDs and their activities and substrate specificities differ from those of NCEDs (Huang et al. 2009a; reviewed by Ohmiya, 2009). The majority of CCDs have been shown to reside in plastids, with the only exception being CCD1, which is located in the cytosol (or possibly the outer membrane of the chloroplast) (reviewed by Auldridge et al. 2006b; Rubio et al. 2008; Baldermann et al. 2010; Brandi et al. 2011).

2.6.2.1 CCD1 and its role in the formation of scent and aroma compounds, as well as in carotenoid turnover

The contribution of CCD1 enzymes to the formation of important apocarotenoid volatiles (e.g. β -ionone, β -cyclocitral, geranylacetone and pseudoionone) in the fruit and flowers of several plant species has been demonstrated in a number of different plant species (Schwartz et al. 2001; Simkin et al. 2004a, b, 2008; Mathieu et al. 2005; Vogel et al. 2008; García-Limones et al. 2008; Huang et al. 2009b). Due to the subcellular location of CCD1, these enzymes do not have direct access to the carotenoids located in the plastids (reviewed by Auldridge et al. 2006b; Rubio et al. 2008; Baldermann et al. 2010; Brandi et al. 2011). It is speculated that plant CCD1s convert the plastid-released apocarotenoids that have arisen through either non-enzymatic oxidative cleavage processes or enzymatic cleavage by other CCDs (CCD4 and/or CCD7). This scenario might explain the multiple cleavage sites and the wide substrate specificity displayed by CCD1

enzymes (Ilg et al. 2010). CCD1 enzymes are involved in the cleavage of the 5,6 (5',6') (Vogel et al. 2008); 7,8 (7',8') (Ilg et al. 2009) and 9,10 (9',10') (Schwartz et al. 2001) double bonds to produce a variety of volatiles. Table 1 provides a list of characterised plant CCD enzymes, with their respective substrates and products.

In planta studies with *Arabidopsis*, tomato and petunia CCD1 mutants or gene silencing transgenics have raised doubts whether the generation of C₁₃ apocarotenoids by CCD1 is the exclusive role for these enzymes (Simkin et al. 2004a, 2004b; reviewed by Auldridge et al. 2006b). Floss & Walter (2009) executed RNA interference (RNAi)-mediated repression of a *Medicago truncatula* CCD1 gene in hairy roots. HPLC results illustrated a differential reduction of C₁₃ and C₁₄ apocarotenoids. This result was conflicting with the hypothesis of a symmetrical cleavage action of CCD1 *in planta*. A prominent colour change to yellow-orange was observed in the mycorrhizal RNAi roots. Analysis performed on the corresponding chromophore indicated a C₂₇ apocarotenoid. These results suggested that C₂₇ derivatives were the main substrates for CCD1 in mycorrhizal roots and not C₄₀ carotenoids as previously thought (Floss et al. 2008; reviewed by Floss & Walter, 2009).

Since CCD1 enzymes do not have direct access to the carotenoids located in the plastids (reviewed by Auldridge et al. 2006b; Rubio et al. 2008; Baldermann et al. 2010; Brandi et al. 2011), it is thought that CCD1 enzymes cleave C₂₇ intermediates that are transported from the chloroplast to the cytosol. During senescence, when the chloroplast membranes disintegrate, CCD1s will however have access to C₄₀ carotenoid substrates (Wise & Hooper, 2007). C₂₇ apocarotenoids have rarely been found in nature, perhaps due to the activity of CCD1s in the plant tissues (Walter et al. 2010) and it is speculated that plant CCD1s also convert the plastid-released C₂₇ apocarotenoids that have arisen through the non-enzymatic oxidative cleavage processes (Ilg et al. 2010). Therefore it has been proposed that CCD1s are not necessarily directly involved in carotenoid maintenance; this hypothesis is supported by their ubiquitous presence, as shown for CCD1s expression in grapevine (Fasoli et al. 2012).

Table 1. A list of plant CCD1s identified, with cleavage sites, substrates and products.

Genes	Specie	Cleavage	Type of Assay	Substrates	Products	Reference	
LeCCD1a	<i>Lycopersicon esculentum</i>	5,6 (5',6')	<i>In vivo and in vitro</i>	phytoene	geranylacetone	Simkin et al. 2004b	
LeCCD1b		9,10 (9',10')		ζ-carotene	pseudoionone	Vogel et al. 2008	
				δ-carotene	6MHO		
				lycopene	α-ionone		
				β-carotene	β-ionone		
				zeaxanthin	3-hydroxy-β-ionone		
PhCCD1	<i>Petunia hybrida</i>	9,10 (9',10')	<i>In vivo</i>	β-carotene	β-ionone	Simkin et al. 2004a	
CmCCD1	<i>Cucumis melo</i>	9,10 (9',10')	<i>In vitro</i>	phytoene	geranylacetone	Ibdah et al. 2006	
				lycopene	pseudoionone		
				β-carotene	β-ionone		
				δ-carotene	α-ionone		
VvCCD1	<i>Vitis vinifera</i>	5,6 (5',6')	<i>In vitro</i>	zeaxanthin	3-hydroxy-β-ionone	Mathieu et al. 2005	
				9,10 (9',10')	lutein	6MHO	Lashbrooke 2010
					lycopene	Pseudoionone;	
					β-carotene	β-ionone	
CsCC1a CsCCD1b	<i>Crocus sativus</i>	9,10 (9',10')	<i>In vitro</i>	β-carotene	β-ionone	Rubio et al. 2008	
FaCCD1	<i>Fragaria ananassa</i>	9,10 (9',10')	<i>In vitro</i>	zeaxanthin	β-ionone	García-Limones et al. 2008	
				lutein	3-hydroxy-β-ionone		
				β-apo-8'-carotenol	3-hydroxy-α-ionone		
ZmCCD1	<i>Zea mays</i>	5,6 (5',6')	<i>In vitro</i>	ζ-carotene	geranylacetone	Vogel et al. 2008	
		9,10 (9',10')		lycopene	pseudoionone		
				δ-carotene	6MHO		
				β-carotene	α-ionone		
				zeaxanthin	β-ionone		
					3-hydroxy-β-ionone		
RdCCD1	<i>Rosa damascena</i>	5,6 (5',6')	<i>In vitro</i>	β-carotene	β-ionone	Huang et al. 2009b	
		9,10 (9',10')		neoxanthin	grasshopper ketone		
				lycopene	6MHO		
OsCCD1	<i>Oryza sativa</i>	7,8 (7',8')	<i>In vitro</i>	lycopene	6MHO	Ilg et al. 2009	
					Pseudoionone		
					geranial		
OfCCD1	<i>Osmanthus fragrans</i>	9,10 (9',10')	<i>In vitro</i>	α-carotene	α-ionone	Baldermann et al. 2010	
				β-carotene	β-ionone		

2.6.2.2 CCD4 and its role in aroma and pigment formation, as well as in carotenoid turnover

From *in vitro* studies, CCD1 and CCD4 enzymes cleave carotenoids at the same 9,10 (9',10') double bond position and have a key role in the formation of β-ionone and other fruit and flower apocarotenoids. CCD4 enzymes seem to be more substrate specific than CCD1, which have a broader substrate tolerance and produce numerous C₁₃ apocarotenoid products (Rubio et al. 2008). According to Huang et al. (2009a), plants generally produce two different forms of CCD4 enzymes.

In planta CCD1 and CCD4 differ in the subcellular location, with CCD1 being cytosolic and CCD4 being plastidial. The presence of plastid target peptides and the confirmed plastid localisation of the CCD4 enzymes allow these enzymes direct access to carotenoid substrates, signifying the role in carotenoid degradation and apocarotenoid synthesis (Rubio et al. 2008; Brandi et al. 2011). AtCCD4, from *A. thaliana* (Vidi et al. 2006; Ytterberg et al. 2006) and CsCCD4 from *C. sativus* (Rubio et al. 2008) have been identified in the plastoglobule proteome. Plastoglobules are structures associated with protein-lipid membranes of thylakoids in chloroplasts. Plastoglobules are involved in the optimisation of photosynthesis, light acclimation and repair (Lundquist et al. 2012), as well as to protect the thylakoid membranes against oxidative stress (Brehelin & Kessler, 2008). The various CCD4-type proteins may thus differ in many respects including their sub-plastidial location (Walter et al. 2010). Brandi et al. (2011) suggested that due to their different subcellular localisation, CCD1s contribute towards volatile production; whereas CCD4s possibly control carotenoid maintenance.

RNA interference studies revealed that suppression of *CmCCD4a* expression contributed to the yellow colour formation in chrysanthemum petals. This suggested that the white colour is a result of the degradation of carotenoids into colourless compounds by *CmCCD4a* (Figure 6). *CmCCD4a* cleaves β -carotene at the 9,10 (9',10') double bond positions resulting in the formation of white petals (Ohmiya et al. 2006, Huang et al. 2009a). Table 2 lists known plant CCD4 enzymes with their respective substrates and products mentioned.

Table 2. A list of known plant CCD4 enzymes, substrates and products.

Gene	Specie	Type of assay	Substrate	Product	Reference
<i>CmCCD4a</i>	<i>Chrysanthemum morifolium</i>	<i>In vitro</i>	β -carotene	β -ionone	Ohmiya et al. 2006
<i>CmCCD4b</i>		<i>In vivo</i>			Huang et al. 2009a
<i>CsCCD4a</i>	<i>Crocus sativus</i>	<i>In vitro</i>	β -carotene	β -ionone	Rubio et al. 2008
<i>CsCCD4b</i>					
<i>RdCCD4</i>	<i>Rosa damascena</i>	<i>In vitro</i>	β -carotene 8'-apo- β -caroten-8'-al	β -ionone	Huang et al. 2009a
<i>MdCCD4</i>	<i>Malus domestica</i>	<i>In vivo</i>	β -carotene	β -ionone	Huang et al. 2009a
<i>AtCCD4</i>	<i>Arabidopsis thaliana</i>	<i>In vitro</i>	8'-apo- β -caroten-8'-al	β -ionone	Huang et al. 2009a

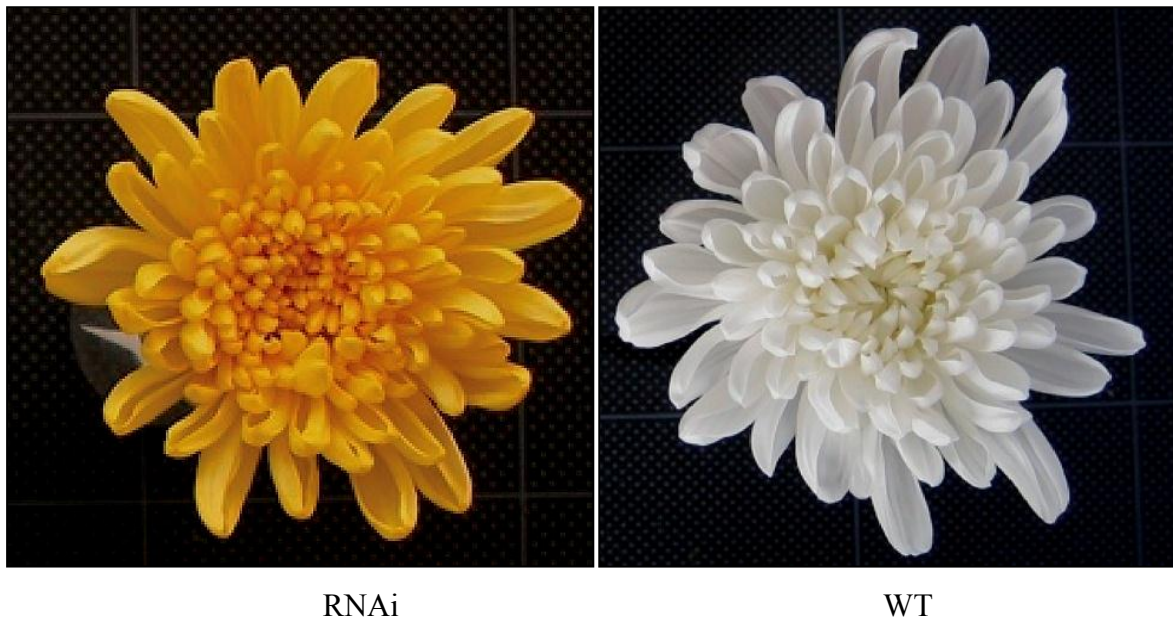


Figure 6 This figure depicts that the suppression of *CmCCD4a* expression contributed to the yellow colour formation in chrysanthemum petals. Chrysanthemum flowers of wild-type ‘Jimba’ (WT) and a transgenic harbouring *CmCCD4a* RNAi (RNAi) are shown (as shown in Ohmiya et al. 2009).

In *Ipomoea* plants, *CCD4* is not involved in the degradation of chromoplast-type carotenoids, but is involved in the degradation of chloroplast-type carotenoids (Goodwin & Britton, 1988; Tai & Chen, 2000; Kishimoto et al. 2005). The carotenoid content in the petals of *Ipomoea* plants is neither related to carotenoid degradation activity nor to the sink capacity of carotenoids, but rather linked to the transcriptional down-regulation of the carotenogenic gene, *CHYB* encoding a β -carotene hydroxylase (Yamamizo et al. 2010).

Potato tubers were analysed in a similar manner as Ohmiya et al. (2006) did with the chrysanthemum petals. Tubers from the white-fleshed cultivar showed higher transcript levels of a *CCD4* gene compared to the yellow-fleshed tubers. Stably transformed RNAi lines of the white-fleshed cultivar with down-regulated *CCD4* expression produced tubers with 2- to 5-fold raised carotenoid content. The carotenoid content was also raised in the petals of the RNAi lines, but not in the leaves, stem or roots (Campbell et al. 2010).

Similar results as seen by Ohmiya et al. (2006) were observed by Brandi et al. (2011) in ‘Redhaven’ peach fruits. In a white-fleshed peach mutant, the *CCD4* transcript accumulated during maturing fruit compared to a yellow-flesh type. The differential expression of *CCD4* was implicated in playing a role in carotenoid accumulation in peach fruits. Citrus *CCD4a* and *CCD4b* showed opposite expression patterns (Pan et al. 2012) and these results were consistent with a previous report by Huang et al. (2009a), demonstrating that *CCD4a* and *CCD4b* have different substrates and thus different biological functions. Citrus *CCD4b*, but not *CCD4a* was down-regulated in citrus (cv Cara Cara), suggesting that *CCD4b* may play a role in lycopene

accumulation in this cultivar rather than CCD4a. Validating the functions of CCD4 enzymes, clarifying their substrate specificities and investigating their expression patterns will shed light on their roles in colour and aroma formation in fruits and flowers (Pan et al. 2012).

The steady state levels of carotenoids are expected to be dependent on the storage capacity of plastids, but also on the balance between biosynthesis and degradation (Ruiz-Sola & Rodríguez-Concepción, 2012). Thus there is continuous turnover of carotenoids and chlorophyll in photosynthetic tissues (Lu & Li, 2008). *In planta*, CCD4s differ in the subcellular location to CCD1 and the confirmed plastid localisation of the CCD4 enzymes allows these enzymes access to carotenoid substrates, signifying a possible role in carotenoid turnover (Rubio et al. 2008; Brandi et al. 2011).

2.6.2.3 CCD7 and CCD8 and their role in strigolactone (SL) formation

CCD7 and CCD8 were first characterised as the two remaining members of the *A. thaliana* NCED/CCD family. AtCCD7 exhibited specific 9,10 (9',10') cleavage activity *in vitro* by converting β -carotene to the C₂₇ compound β -apo-10'-carotenal and the C₁₃ compound β -ionone. When AtCCD7 was co-expressed in *E. coli* with AtCCD8, an additional cleavage product was identified, namely β -apo-13-carotenal. This was not identified when AtCCD8 was expressed on its own. The conclusion was that β -apo-13-carotenal was formed by a secondary cleavage of the C₂₇ compound β -apo-10'-carotenal, formed by AtCCD7, at the 13,14 (13',14') double bond position (Schwartz et al. 2004; Walter et al. 2010). *In vivo* studies showed the formation of β -apo-13-carotenone from all-*trans*- β -apo-10'-carotenal by CCD8 enzymes from several plant species (Alder et al. 2008). The structure of β -apo-13-carotenone has very little similarities with that of SLs, therefore it was questionable whether β -carotene was the initial substrate in the pathway for SL biosynthesis. Moreover, when a rice *ccd8* mutant was treated with β -apo-13-carotenone, the wild-type phenotype was not restored (Alder et al. 2012).

Alder et al. (2012) demonstrated that D27 is a β -carotene isomerase that converts the all-*trans*- β -carotene into a 9-*cis*- β -carotene, which is subsequently cleaved by CCD7 into a 9-*cis*-configured aldehyde. CCD8 then incorporates three oxygen molecules into 9-*cis*- β -apo-10'-carotenal and rearranges it molecularly to form carlactone (Figure 7). Additional shoot branching was observed in *d27*, *ccd7* and *ccd8* mutants in the absence of carlactone. With the addition of *in vitro* produced carlactone to rice mutants *d27*, *ccd7* (*htd-1*) and *ccd8* (*d10*), the wild-type phenotype was restored, indicating that carlactone is a likely SL precursor (Wang & Li, 2011; Alder et al. 2012).

SLs are a fairly new class of plant hormones which are involved in several aspects of plant growth and development. They were originally identified as germination stimulants for root parasitic plant seeds of the family Orobanchaceae, such as *Striga*, *Phelipanche* and *Orobanche* spp (Cook et al. 1966; Bouwmeester et al. 2003). SLs are exuded by plant roots and act as host detection signals for symbiotic interactions with arbuscular mycorrhizal fungi (AM), stimulating their metabolism and hyphal branching (Akiyama et al. 2005; Parniske, 2008). The most extensively characterised role of SLs is their involvement in the inhibition of shoot branching (reviewed by Dun et al. 2009; Domagalska & Leyser, 2011; Waters et al. 2012). SL-response and SL-deficient mutants have illustrated several additional roles, namely enhanced lateral (Stirnberg et al. 2002; Arite et al. 2007; Kapulnik et al. 2011) and adventitious root development (Rasmussen et al. 2012), reduced stature and suppression of cambium ring development in the main stem (secondary development) (Agusti et al. 2011).

Experiments using the synthetic SL, GR24, demonstrated that this hormone (or a derived compound) could partially restore the wild-type branching phenotypes and bud outgrowth in *ccd7* and *ccd8* mutants of pea, rice and *Arabidopsis* (Gomez-Roldan et al. 2008; Umehara et al. 2008). Studies using Fluridone, an inhibitor of carotenoid biosynthesis, have illustrated that carotenoid biosynthesis is necessary for normal levels of SLs (Ito et al. 2010).

A. thaliana and petunia (*Petunia hybrida*) are important model plants in which axillary branching has been studied. In *P. hybrida*, mutation of the *PhCCD7* gene had a less severe branching phenotype than mutation of the *PhCCD8* gene. Analysis of expression of *PhCCD7* and *PhCCD8* in the wild type, mutants and grafted petunia suggested that *PhCCD7* and *PhCCD8* are co-ordinately regulated (Drummond et al. 2009). Recombinant AtCCD7 cleaves multiple carotenoid substrates (Booker et al. 2004; Schwartz et al. 2004). The expression analysis of *AtCCD7* revealed the transcripts were more abundant in seeds, than other organs. The robust expression of *AtCCD7* in mature seeds is in contrast to previous findings, which illustrated its highest expression to be in roots (Liang et al. 2011).

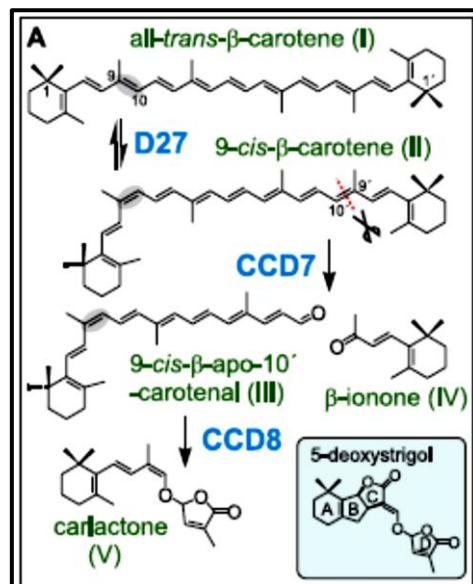


Figure 7 The pathway to carlactone. D27 catalyses the isomerisation of the 9,10 double bond (shaded) in all-*trans*-β-carotene (I; C₄₀), forming 9-*cis*-β-carotene (II) that is cleaved by CCD7 at the 9',10' position to form 9-*cis*-β-apo-10'-carotenol (III, C₂₇) and β-ionone (IV, C₁₃). CCD8 cleaves III to carlactone (V) that contains the D ring and the enol ether bridge of strigolactones such as 5-deoxystrigol (inset) (Alder et al. 2012).

2.7. CCDs in grapevine (*V. vinifera*): current knowledge

In grapevine, two NCED genes *VvNCED1* and *VvNCED2* were described by Soar et al. (2004) to possess similar characteristics to other NCEDs. Zhang et al. 2009 analysed the expression of *NCED1* isolated from *V. vinifera* (*VvNCED1*) during development and ripening. *VvNCED1* was only expressed at the initial stages of ripening, when the ABA was highest. Young et al. (2012) illustrated that *VvNCED2* was down regulated from the green to the ripe stages of berry development and the expression of *VvNCED1* (known as *VvNCED3* in the study) peaked around the véraison stage of berry development.

Mathieu et al. (2005) identified and isolated a CCD1 (*VvCCD1*) from *V. vinifera* L. The recombinant expression of *VvCCD1* confirmed that the gene encoded a functional CCD. Two *in vitro* substrates for *VvCCD1* were identified, namely zeaxanthin and lutein, generating the volatile flavour compound 3-hydroxy-β-ionone (C₁₃ norisoprenoid) and a C₁₄-dialdehyde (Mathieu et al. 2005, 2007). Lashbrooke (2010) identified two additional *in vitro* substrates for *VvCCD1*, lycopene generating MHO and pseudoionone and β-carotene generating β-ionone. Lashbrooke (2010) tried to determine the *in planta* roles of *VvCCD1* by generating *V. vinifera* L. lines that were transgenically altered for *VvCCD1* expression (overexpressed and silenced). Only photosynthetically active leaves were analysed, no reproductive tissues or senescing leaves were analysed. A weak correlation between gene expressed and apocarotenoid formation was observed, but correlations were found amongst apocarotenoid pools. The author suggested that a division between the availability of the respective carotenoid substrates existed, which was based on either enzyme access or enzyme-substrate specificity. The author hypothesised that the

plastid-localised enzymes differentially cleaved these carotenoids, resulting in apocarotenoids which were then transported to the cytosol, which were further cleaved by VvCCD1. The author also suggested that future studies should include the reproductive organs of grapevine. Lashbrooke (2010) revealed that the *in planta* role of VvCCD1 was distinct from the demonstrated *in vitro* functions. Expression analysis done by Young et al. (2012) illustrated that a number of genes change at or around the véraison stage of berry development including VvCCD1 expression that peaked at véraison.

Guillaumie et al. (2011) identified and isolated a CCD4 (VvCCD4a) from *V. vinifera* L., but was unable to confirm functionality of VvCCD4a. Expression analysis of VvCCD4a demonstrated the up-regulation of VvCCD4a during the last stage of berry ripening which was confirmed by Young et al. (2012) that also illustrated the up-regulation of VvCCD4a and VvCCD4b during berry ripening.

Prior to the study described in this thesis, no additional expression data was available for grapevine CCDs, but most recently Fasoli et al. (2012) reported a genome-wide transcriptomic atlas of grapevine based on 54 sample tissues spanning most tissue types in grapevine. From this analysis it was revealed that VvCCD1 was present in 54/54 and VvCCD4s in more than 40/54 of the tissue samples tested.

2.8. Conclusion

Plant carotenoid cleavage dioxygenases catalyse the oxidative cleavage of a number of carotenoid and/or apocarotenoid substrates, resulting in the formation of apocarotenoids that perform several biological roles in plants, including hormones, pollinator attractants and flavour and aroma compounds. Carotenoids are an attractive resource for the industrial production of bioactive substances, namely aroma (β -ionone and β -damascenone), colourants (safranal) and vitamins (Vitamin A). Apocarotenoid biogenesis and the enzymes involved have become a very important aspect of research. The phytohormone ABA; plays an important role in the regulation of seed development, drought resistance and sugar sensing. Another carotenoid-derived phytohormone, SL is involved in branch inhibition and is a modulator of root development. Plants release volatile apocarotenoids, namely damascenone and β -ionone, which are essential aroma compounds in roses, grapes, tobacco, tea and wine. These apocarotenoid volatiles are emitted by many flowers and vegetative tissues, as well as contribute towards the floral and fruity characteristics of wine. The biological roles of the genes involved in apocarotenoid biosynthesis have been studied in a number of plant species *in vitro*, but whether this is the enzymes' primary roles *in planta* is still unclear.

2.9. References

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Chapter 3

Research results

Functional analysis of the carotenoid cleavage
dioxygenase family of *Vitis vinifera*.

3.1. Introduction

Carotenoids are isoprenoid pigments that are present in the membranes of all phototrophic as well as many heterotrophic organisms (Goodwin, 1980). In plants, their presence is revealed by the rich colours of fruits, flowers and storage organs in the yellow to red spectrum. Their structure is formed by a characteristic linear C₄₀ molecular backbone containing up to the 15 conjugated double bonds. Carotenoids have different roles, depending on which type of plastid they are synthesised and present in. Carotenoids in photosynthetically active chloroplasts protect the photosynthetic apparatus from photo-oxidation and are part of the light-harvesting and reaction centre complexes (Demmig-Adams & Adams, 1996; Pogson et al. 1998; Havaux & Niyogi, 1999; Park et al. 2002; Ahrazem et al. 2010). Carotenoids present in chromoplasts play a role in attracting seed-dispersal and pollination agents (Zhu et al. 2010), whereas those found in etioplasts and leucoplasts are essential in the formation of the phytohormones strigolactone (SL) and abscisic acid (ABA) (Gomez-Roldan et al. 2008; Umehara et al. 2008).

Apocarotenoids are a class of terpenoid compounds generated by oxidative cleavage of carotenoids and apocarotenoids, either by enzymatic cleavage (by carotenoid cleavage dioxygenases or non-specific enzymes such as lipoxygenases and peroxidases (Carail & Caris-Veyrat, 2006)) or non-enzymatic cleavage (photochemical processes or other conditions of oxidative stress) (Ramel et al. 2012). This structurally diverse group of compounds is widely distributed throughout nature. The assortment of apocarotenoids results from the large number of carotenoid substrates (more than 700 have been identified), variations in cleavage site and modification(s) subsequent to cleavage (Schwartz et al. 2001). The best studied apocarotenoid is the phytohormone ABA, which plays an important role in the regulation of seed maturation and dormancy, drought resistance and sugar sensing (Schwartz et al. 2003; Taylor et al. 2005). Several examples have been described where apocarotenoids act as repellents, chemo-attractants, growth stimulators and inhibitors (Bouvier et al. 2003).

Two types of carotenoid dioxygenases have been identified in plants: *9-cis* epoxy-carotenoid dioxygenases (NCEDs) and carotenoid cleavage dioxygenases (CCDs) (Tan et al. 2003; Bouvier et al. 2003; Simkin et al. 2004a; reviewed by Auldrige et al. 2006a). The NCEDs are involved in the biosynthesis of the plant hormone ABA. *CCD1s* encode for enzymes that cleave a broad range of carotenoids forming volatile aroma compounds; *CCD4s* encode enzymes catalysing the cleavage of carotenoids forming the aroma and pigment compounds and *CCD7s* and *CCD8s* encode enzymes that catalyse the sequential cleavage of carotenoids forming SL, which is involved in the inhibition of shoot branching (Matusova et al. 2005; Auldrige et al. 2006b;

Gomez-Roldan et al. 2008; Umehara et al. 2008; Domagalska & Leyser, 2011; Waters et al. 2012).

CCD1 is, after the NCEDs, the most studied CCD due to its role in the formation of C₁₃ apocarotenoid-based flower scent, as well as in wine and fruit aroma biosynthesis (Simkin et al. 2004a; Simkin et al. 2004b; Mathieu et al. 2005). CCD1s have been shown to catabolise an extensive range of 9-*cis*- and all-*trans*-carotenoids and epoxy-carotenoids (Schwartz et al. 2001; Simkin et al. 2004a; Ibdah et al. 2006; Huang et al. 2009a). Previous studies have shown that a CCD1 isolated from *Vitis vinifera* (VvCCD1) was able to cleave multiple carotenoid substrates *in vitro*. VvCCD1 cleaved zeaxanthin and lutein to produce 3-hydroxy- β -ionone (Mathieu et al. 2005). Two additional substrates were identified by Lashbrooke (2010), namely lycopene and β -carotene producing 6-methyl-5-hepten-2-one (MHO) and pseudoionone and β -ionone, respectively.

Plants generally produce two CCD4 isoforms, namely CCD4a and CCD4b that may have different expression patterns and/or substrate specificities and thus different biological functions in plants (Huang et al. 2009b). *In planta*, CCD1 and CCD4 differ in their respective subcellular location: CCD1 is cytosolic (or associated with the outer membrane of the chloroplast) and CCD4 is plastidic. The presence of plastid target peptides allows the CCD4 enzymes access to carotenoid substrates, indicating a possible role in carotenoid degradation and apocarotenoid synthesis (Rubio et al. 2008; Brandi et al. 2011). Brandi et al. (2011) suggested that due to their different subcellular localisation, CCD1s contribute towards volatile production whereas CCD4s possibly controls carotenoid maintenance.

VvCCD4a, VvCCD4b, VvCCD4c, VvCCD4d (Ahrazem et al. 2010), VvCCD7 and VvCCD8 from *V. vinifera* were identified *in silico*, but none have been functionally characterised (Vallabhaneni et al. 2010). The specific aims of this study were to isolate putative VvCCD4, VvCCD7 and VvCCD8 genes from *V. vinifera* and illustrate their functionality. VvCCD4a and VvCCD4b were isolated and shown to be functional, but despite several attempts, VvCCD4c, VvCCD4d, VvCCD7 and VvCCD8 were not successfully isolated. VvCCD4a, VvCCD4b, as well as the previously isolated VvCCD1, were heterologously produced in carotenoid-accumulating *Escherichia coli* strains to demonstrate functionality and establish their respective substrates preferences/specificities, after optimising the test system. These results, in combination with the expression patterns of the VvCCD gene family in grapevine shed light on their roles *in planta*.

3.2. Materials and methods

3.2.1. Plant material

Fresh plant material used for gene isolation attempts and expression analysis was harvested from field-grown *Vitis vinifera* L. cv. Pinotage at Welgevallen experimental farm (Stellenbosch University, South Africa). Green, véraison and ripe berries, as well as leaf and flower material were harvested. The harvested material was immediately frozen in liquid nitrogen, ground to a fine powder using a mortar and pestle and stored at -80°C until use in expression analysis and amplification for gene isolation attempts.

Dormant Pinotage cuttings (300-400 mm long) were collected in winter and stored at 4°C until required. To activate budburst, the cuttings were soaked in distilled water overnight. Each cutting was pruned to remove the basal node, with two nodes remaining for budburst. The cuttings were placed in moist perlite and kept in low light conditions for 10 days in a growth room (25°C). After 10 days the cuttings were moved to a greenhouse with natural sunlight and a mean temperature of 25°C. The roots that developed from the cuttings were rinsed and harvested after approximately a month and stored at -80°C as source material to extract root RNA for possible isolation of *VvCCD7* and *VvCCD8*.

3.2.2. In silico analyses

The National Center for Biotechnology Information (NCBI) Entrez search and retrieval system was used to obtain the nucleotide and protein sequences for the putative *VvCCDs* from the Genbank databases (<http://www.ncbi.nlm.nih.gov/Entrez/>). Alignment to sequences in the Genbank database was performed using the relevant BLAST algorithm (Altschul et al. 1990). Comparative genomics (gene structure prediction and homologue/orthologue retrieval) was performed using two database systems, namely Plaza (http://bioinformatics.psb.ugent.be/plaza_v1/), as well as Genoscope (<http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/>) using orthologues from *Arabidopsis thaliana*.

3.2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from roots, leaves, flowers and berries using a cetyltrimethylammonium bromide (CTAB) extraction method described in Chang et al. (1993). RNA samples were treated with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) as described by the supplier. After DNase-treatment, RNA was purified by means of an RNeasy Plant Mini Kit (Qiagen), for further removal of genomic DNA contamination. First-strand cDNA was synthesised using 1 µg of total RNA as template in a 20 µL reaction volume, using SuperScript III Platinum first strand synthesis system as described by the supplier (Invitrogen).

3.2.4. Isolation of putative *VvCCD*-encoding genes

All primer sequences used are reported in Table 1 and all plasmids used and generated are shown in Table 2. Full-length and real time primers were designed using Primer Express 3 based on the sequences identified from the *in silico* analysis and used to PCR screen cDNA pools for *VvCCD4a*, *VvCCD4b*, *VvCCD4c*, *VvCCD7* and *VvCCD8*. The primers were designed at the ATG and STOP codons and specific enzyme restriction sites were added. The cDNA pools were screened and those that produced the expected amplicons were used for subsequent gene isolation. The cDNA fragments of *VvCCD1*, *VvCCD4a* and *VvCCD4b* were PCR-amplified. The resultant PCR amplicons were cloned into the pGEMt-Easy vector system according to the specifications of the supplier (Promega) and yielding the plasmids as outlined in Table 2. The recombinants were subjected to sequencing to confirm the sequence (integrity) of the inserts.

Table 1 The primer sequences used for PCR and Real-time PCR amplification of the putative coding regions of *VvCCD1*, *VvCCD4a*, *VvCCD4b*, *VvCCD4c*, *VvCCD4d*, *VvCCD7*, *VvCCD8* and *EF1a*. The restriction sites which were added are underlined.

	Primers	Sequences (5' to 3')	Amplicon size	T _m (°C)
CCD1	VvCCD1_5'	<u>CATATGGCGGAGAAGGAGGAG</u>	1715bp	64
	VvCCD1_3'	<u>AGATCTTCAAAGTTTTGCTTGTCTTT</u>		57
CCD4a	VvCCD4a_5'	<u>CATATGGACGCCTTCTCTTCCT</u>	1722bp	58
	VvCCD4a_3'	<u>AGATCTGTCAGAGTCCTTTAATGTCCCT</u>		61
CCD4b	VvCCD4b_5'	<u>CATATGAACCCTTTGTTTTGCCCT</u>	1770bp	61
	VvCCD4b_3'	<u>GGATCCTCAGTCCATTTGAGATCACA</u>		64
CCD7	VvCCD7_5'	<u>CATATGCAGGCCAAACCTTT</u>	1800bp	50
	VvCCD7_3'	<u>AGATCTCTACTCTTTGGGGGCC</u>		59
CCD8	VvCCD8_5'	<u>CATATGGCTTCTACACAATTTTCTTCT</u>	1692bp	54
	VvCCD8_3'	<u>AGATCTCTATTTCTTGGGAACCCA</u>		54
CCD1	RTVvCCD1_5'	CCATACGGTTTTTCATGCCTTC	97bp	60
	RTVvCCD1_3'	TGCATACCTTACATGGCGGTC		60
CCD4a	RTVvCCD4a_5'	TGTGGGAAGCAGGATGTATGG	66bp	59
	RTVvCCD4a_3'	GGCTCCCTTGCCACGAA		59
CCD4b	RTVvCCD4b_5'	AAGCGGGAAATCAAGGTTTCATAG	82bp	59
	RTVvCCD4b_3'	CCTTGIGGGCAGCTTCACA		59
CCD4c	RTVvCCD4c_5'	CCATTGATAAGGAAGGCTCCA	61bp	59
	RTVvCCD4c_3'	GTGGGATCGGTCATGGAGAA		59
CCD4d	RTVvCCD4d_5'	TTTGAGAGGAGAACCCTTGTTTG	61bp	59
	RTVvCCD4d_3'	TGTACCCGTCATCCTCCTCACT		59
CCD7	RTVvCCD7_5'	GAAGACGACGGTTACCTTCTGGTA	816bp	57
	RTVvCCD7_3'	CAAGTGATCAATCTCTCCTATCTCT		57
CCD8	RTVvCCD8_5'	GCCCTTGTAACCTCCCCAACA	152bp	54
	RTVvCCD8_3'	ATCGAACCAGTTCTTTGCCTTCT		53
EF1a	EF1a_5'	TCTGGAGCCAAGGTCACCAA	150pb	60
	EF1a_3'	GCTGTACCCAAAACCTGCCAAAA		60

3.2.5. Real-time PCR analysis

Expression analysis of *VvCCD1*, *VvCCD4a*, *VvCCD4b*, *VvCCD4c*, *VvCCD4d*, *VvCCD7* and *VvCCD8* was performed via Real-time PCR. The elongation factor 1 α (*EF1 α*) was used as a “house-keeping” gene to normalise expression. The expression of *EF1 α* was shown to be constant during berry development (Terrier et al. 2005; Reid et al. 2006; Guillaumie et al. 2011). Primers were designed using Primer Express 3.0 (Table 1), yielding the various primer sets starting with “RT”. The reaction mixture contained 2 μ L of a 1:2 dilution of the first-strand cDNA synthesis reaction. Real time PCR was performed using an ABI 7500 Real-time PCR system and the KAPA SYBR FAST Universal qPCR kit (KapaBiosystems). The thermal cycling conditions consisted of the initial denaturation step at 95°C for 3 minutes, followed by 40 cycles at 95°C for 3 seconds and then 59°C for 32 seconds. All PCR reactions consisted of three technical replicated. The relative expression was calculated using the equation describe by Pfaffl (2001), where *E* is the PCR efficiency and CP is the cycle number at which the florescence crosses the base line.

$$(E_{\text{target}})^{\Delta\text{CP}_{\text{target}}(\text{control-sample})} / (E_{\text{reference}})^{\Delta\text{CP}_{\text{reference}}(\text{control-sample})}$$

To calculate the efficiency of each primer pair, Real-time PCR was performed on a dilution series of template DNA and the CP values were plotted relative to the template concentration. The standard curves were used to determine the PCR efficiency according to the equation described by Pfaffl (2001), $E=10^{(-1/\text{slope})}$.

3.2.6. Cloning of putative *VvCCD*-encoding genes into bacterial expression vectors

The pGEMt-constructs were digested with *Nde*I and *Bgl*II (for *VvCCD4a*); *Nde*I and *Bam*HI (for *VvCCD4b*) and partial digestion with *Nde*I and then *Pst*I (for *VvCCD1*). The digested fragments were separated on a 1% (w/v) agarose gel and purified by Wizard[®]SV Gel and PCR Clean-Up System (Promega), according to manufacturer’s instructions. The purified fragments were ligated into pTWIN1-vectors which were digested with the *Nde*I and *Bam*HI (for *VvCCD4a* and *VvCCD4b*) and *Nde*I and *Pst*I (for *VvCCD1*) to yield pTWIN1-*VvCCD1*; pTWIN1-*VvCCD4a* and pTWIN1-*VvCCD4b* (Table 2). The recombinants were subjected to sequencing to confirm the sequence of the inserts. The recombinants were digested with *Eco*47III, the ends dephosphorylated using FastAP thermosensitive alkaline phosphatase (Fermentas), according to supplier’s instructions and a pRARE cassette was ligated into the pTWIN1-constructs (Table 2).

Table 2 Description of the plasmids used in this study

Name of Plasmid	Description	Reference
pGEMt-VvCCD1	<i>CCD1</i> isolated from <i>V. vinifera</i> and cloned into pGEMt-easy cloning vector.	This study
pGEMt-VvCCD4a	<i>CCD4a</i> isolated from <i>V. vinifera</i> and cloned into pGEMt-easy cloning vector.	This study
pGEMt-VvCCD4b	<i>CCD4b</i> isolated from <i>V. vinifera</i> and cloned into pGEMt-easy cloning vector.	This study
pTWIN1-VvCCD1	<i>VvCCD1</i> was isolated from pGEMt-VvCCD4a and cloned into pTWIN1	This study
pTWIN1-VvCCD4a	<i>VvCCD4a</i> was isolated from pGEMt-VvCCD4a and cloned into pTWIN1	This study
pTWIN1-VvCCD4b	<i>VvCCD4b</i> was isolated from pGEMt-VvCCD4a and cloned into pTWIN1	This study
pTWIN1-VvCCD1-RARE	pRARE cassette was ligated into pTWIN1-VvCCD1	This study
pTWIN1-VvCCD4a-RARE	pRARE cassette was ligated into pTWIN1-VvCCD4a	This study
pTWIN1-VvCCD4b-RARE	pRARE cassette was ligated into pTWIN1-VvCCD4b	This study
pAC-PHYT	pAC-plasmid which contains the necessary precursors required for the formation of the carotenoid phytoene	Cunningham et al. 1994
pAC-ZETA	pAC-plasmid which contains the necessary precursors required for the formation of the carotenoid ζ -carotene	Cunningham et al. 1994
pAC-NEUR	pAC-plasmid which contains the necessary precursors required for the formation of the carotenoid neurosporene	Cunningham et al. 1994
pAC-LYC	pAC-plasmid which contains the necessary precursors required for the formation of the carotenoid lycopene	Cunningham et al. 1994
pAC-EPSILON	pAC-plasmid which contains the necessary precursors required for the formation of the carotenoid ϵ -carotene	Cunningham & Gantt, 2005
pAC-BETA	pAC-plasmid which contains the necessary precursors required for the formation of the carotenoid β -carotene	Cunningham et al. 1996
pAC-ZEAX	pAC-plasmid which contains the necessary precursors required for the formation of the carotenoid zeaxanthin	Cunningham et al. 1994

3.2.7. Transformation of carotenoid accumulating *E. coli* strains with *VvCCD* expression vectors

pTWIN1-VvCCD1-RARE, pTWIN1-VvCCD4a-RARE and pTWIN1-VvCCD4b-RARE plasmids were introduced into *E. coli* strains capable of accumulating phytoene, ζ -carotene, neurosporene, lycopene, ϵ -carotene, β -carotene and zeaxanthin (described in Table 2). These plasmids were obtained from F. X. Cunningham (Department of Cell Biology and Molecular Genetics, University of Maryland, MD, USA) and are described in Cunningham & Gantt, (2007). Cultures were grown overnight at 37°C in Luria Burtani (LB) broth containing 100 $\mu\text{g}\cdot\text{mL}^{-1}$ ampicillin, 34 $\mu\text{g}\cdot\text{mL}^{-1}$ chloroamphenicol and 12.5 $\mu\text{g}\cdot\text{mL}^{-1}$ tetracycline. Freeze cultures were prepared and stored at -80 °C until required for HS-SPME-GC-MS analysis and/or UPLC analysis.

3.2.8. *Determination of volatile apocarotenoids from bacterial culture headspace*

Bacterial cultures containing pTWIN1-VvCCD1-RARE, pTWIN1-VvCCD4a-RARE and pTWIN1-VvCCD4b-RARE plasmids (see section 3.2.7) were used in the analysis and the pTWIN1 containing culture was used as negative control. Significant optimisation of the culturing conditions, protein (CCD) production, culture sample size, addition of co-factors and anti-oxidants were undertaken to ensure an optimised method where the enzymatic activities of the CCDs could be accurately monitored. Moreover, the carotenoid substrates produced by the bacterial cultures were profiled and quantified by UPLC analysis (see section 3.2.10) prior to the induction of CCD enzyme production. This experimental plan ensured profiling/quantification of the carotenoid substrates as well as the volatile products that would be formed from VvCCD1, VvCCD4a and VvCCD4b, whereas the conditions were optimised to ensure optimal enzymatic activity, while limiting non-enzymatic cleavage of the carotenoid substrates. The optimisations and their results regarding the production of the heterologous volatile apocarotenoid production and profiling are described in Addendum A to Chapter 3. These optimisations led to the following method: An overnight culture (5 mL) was used to inoculate 32 mL of LB, containing the appropriate antibiotics at an OD_{600nm} of 0.1. The cultures were incubated in the dark, gently shaking at room temperature until an OD_{600nm} of 0.6 was reached. 100 μ M of the carotenoid inhibitor diphenylamine (DPA) was added and the cultures were incubated at room temperature for an additional two hours in the dark, to prevent the formation of any additional carotenoids. After two hours, 8 mL of each culture was removed, flash frozen and stored at -80°C for future carotenoid substrate profiling and quantification by UPLC (see section 3.2.10 and Addendum B to Chapter 3). The remaining 24 mL of each culture was used to harvest the cells. The cells were resuspended in 6 mL of LB containing the appropriate antibiotics, 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to induce protein production, 6 mM ascorbate (as anti-oxidant), 5 μ M ferrous sulphate (as CCD co-factor), 200 U/mL catalase (as anti-oxidant) and 1000 μ g/L of α -terpineol (as internal standard). The cultures were transferred to a 20 mL SPME vial. The vials were sealed with Bi-metal® crimp seals with 20 mm silicone/polytetrafluoroethylene (PTFE) septa (Brown Chromatographic supplies, Wertheim, Germany). The samples were subsequently incubated in the dark, gently shaking at room temperature for 16 hours to allow CCD production and cleavage of the carotenoid substrates. After 16 hours, 1 mL of each culture was removed from the 20 mL capped SPME vial using a syringe to determine the OD_{600nm} . 5 mL of 5 M NaCl was added to each sample to terminate the enzyme reactions. Each strain was grown in triplicate and from each culture one sample was prepared; the samples were placed simultaneously on the GC-MS instrument.

HS-SPME extraction was performed using a CTC CombiPal auto sampler equipped with the SPME option (CTC Analytics, Switzerland). Extraction conditions were as follows: after incubation at 40°C for 2 minutes SPME extraction was performed for 15 minutes under constant agitation by exposing a divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME grey fibre (Supelco, Bellefonte, PA) to the headspace. The fibre was then desorbed and analytes subsequently injected onto the GC column using a split/splitless injector, operated at 240°C, in splitless mode for 2 minutes. Thereafter the fibre was left in the injector for a further 20 minutes at 270°C for thermal desorption of the fibre under a purge flow of 60 mL/min. Separation of compounds was achieved on a DB-FFAP column (60 m x 0.25 mm x 0.5 µm) on an Agilent 6890 gas chromatograph coupled to an Agilent 5975C mass spectrometer (Agilent Technologies, Little Falls, Wilmington, USA). The helium carrier gas flow through the column was 1.2 mL/min and the oven programmed from 40°C (5 min hold), ramped at 10°C/min to 230°C (held for 2 min), with a post run at 240°C (held for 2 min). The total run time was 30 minutes.

The mass spectrometer (MS) was operated in electron impact (EI) mode (70 eV) using Selected Ion Monitoring (SIM), simultaneously acquiring scan data as well. In SIM mode the m/z fragments which were monitored are listed in Table 3. Compound identification was performed by comparisons of the retention times with that of the authentic standards (listed in Table 3) as well and the NIST2005 mass spectral library (National Institute of Standards, USA). Addendum A to Chapter 3 describes the calibration curves that were created using the authentic standards mentioned in Table 3. Quality controls were run prior to the analysis of the first sample and after the analysis of the last sample. The volatile apocarotenoids were quantified and expressed as normalised values to the internal standard (α -terpineol) and the OD_{600nm} and expressed relative to the pTWIN1 (negative control). T-tests were used to determine statistical significance.

Table 3. A list of the authentic standards used in HS-SPME-GCMS analysis, as well as the m/z fragments of each that were monitored.

Authentic standard	m/z fragments monitored	Approximate Retention time (min)
α -terpineol	59, 93, 136	21.75
6-methyl-5-hepten-2-one	69, 111, 126	16.95
geranylacetone	69, 136, 121	23.5
α -ionone	121, 136, 192	23.75
β -ionone	136, 177, 192	24.75
pseudoionone	81, 109, 135	25.85 and 27.05

3.2.9. HPLC analysis of carotenoids from plant material

Carotenoid extraction, profiling and quantification from grapevine organs, including leaf, flower, and developmental stages of berry ripening was performed as described in Lashbrooke et al. (2010).

3.2.10. UPLC analysis of carotenoid substrates present in bacterial cultures

UPLC analysis was performed to profile and quantify the carotenoid substrates present at the point of CCD induction. The 8 mL aliquot of each bacterial culture sampled and flash frozen prior to GC-MS analysis (see section 3.2.8) was removed from storage at -80°C and defrosted in the dark at room temperature. The cells were harvested by centrifugation at 5000 g for 5 minutes and resuspended in 80 µL of extraction buffer (1 M NaCl and 50 mM Tris-HCL (pH 8.0)). 320 µL of 100% Methanol and 2 µg.mL⁻¹ of β-apo-carotenol (as internal standard) were added. The samples were vortexed at 8°C for 30 minutes. 320 µL of ethyl acetate was added and the samples were vortexed at 8°C for 5 minutes. 400 µL of extraction buffer was added and the samples were vortexed (as before). The samples were then centrifuged for 5 minutes at 13 000 g. 150 µL of the ethyl acetate organic phase was aliquoted into an amber HPLC vial and crimp sealed.

A Waters ACQUITY system equipped with a Diode Array Detector (DAD) was used for UPLC analysis, scanning from 280 to 700 nm. To separate the different pigments, a Waters UPLC BEH Shield RP₁₈ (2.1 mm x 100 mm, 1.7 µm) column and a Waters UPLC BEH guard cartridge (2.1 mm x100 mm, 1.7 µm) were used. The temperature of the column was set at 25°C and an injection volume of 5 µL was used. The mobile phase consisted of water containing 5% (v/v) acetonitrile and 0.1% (v/v) formic acid (solvent A) and a mixture of acetonitrile/methanol (80/20, v/v) in 0.1% (v/v) formic acid (solvent B). The flow rates and gradients used to achieve separation of the pigments are described in Addendum B to Chapter 3. The control of the instrument, the acquisition and processing of the generated data were done using Empower 2 software from Waters.

UPLC-MS was performed using a Water ACQUITY UPLC system connected to a DAD and a Waters Synapt G2 mass spectrophotometer with the same column; mobile phase and gradient separation were as described above. The major carotenoids extracted from the bacterial cultures were detected using atmospheric pressure chemical isomeration (APCI) in the positive mode. The capillary voltage was set to 3 kV, the corona pin was 8.0 µA and the cone voltage was at 30 V. The temperature of the probe was set at 580°C. UPLC-MS analysis data, comparison between retention times of authentic standards of β-carotene (Fluka Chimie) and zeaxanthin (Sigma-Aldrich) and the visible spectra of the carotenoids described in literature was used to confirm the

peak identity. The quantification of the peaks was done using calibration standards (external) based on areas. Each pigment was normalised to the internal standard concentrations obtained from mock internal standard values. β -apo-caroten-8-al (98% purity) obtained from Fluka Chimie (Buchs, Switzerland) was used as the internal standard. The pigments, for which no authentic standards were available, were sent for MS analysis and the molecular weight was used to quantify the peaks observed (Addendum B to Chapter 3). The data was expressed as percentage of the compounds present.

3.3. Results

3.3.1. Isolation of grapevine CCD encoding genes

Comparative genomics (gene structure prediction and homologue/orthologue retrieval) was performed using Plaza, as well as Genoscope. The gene IDs and accession numbers are listed in Table 5 for *VvNCEDs*, *VvCCD1*, putative *VvCCD4s* and putative *VvCCD7* and *VvCCD8*. Four putative *CCD4* genes were identified in *V. vinifera*. *VvCCD4a* and *VvCCD4b* genes are located in chromosome 2 and separated by 26.9 kb and showed 73% identity to each other on a protein level. Putative *VvCCD4c* and *VvCCD4d* gene fragments are located on chromosome 16 and showed 97% identity to each other in 677 of the nucleotides.

The putative *CCD7* and *CCD8* genes from *V. vinifera* were identified on chromosomes 15 and 4, respectively. A number of putative CCD genes were also identified, but were found to have no similarity to identified CCD orthologues, as several were too small or were truncated sections of putative *VvCCD* genes. The predicted amino acid sequences of the *VvCCD* orthologues were used to generate a phylogenetic tree using ClustalW (<http://www.clustal.org>) algorithm-based AlignX module in Figure 1.

Table 5. The Plaza gene IDs, Genoscope accession numbers, Putative CCD gene names, chromosome position, as well as size of the putative genes in *V. vinifera*.

Gene ID		Accession number	Putative gene	Chromosome number	Chromosome position	Length (aa)
VV02G12270	*	GSVIVT00001163001	<i>CCD4a</i>	2	17383373-17385172	573
VV02G12290	*	GSVIVT00001161001	<i>CCD4b</i>	2	17412303-17414072	589
VV04G03460		GSVIVT00032430001	unknown	4	2860469-2860832	79
VV04G03480		GSVIVT00032432001	unknown	4	2861897-2863580	263
VV04G03500		GSVIVT00032434001	unknown	4	2871209-2875102	518
VV04G03520		GSVIVT00032436001	unknown	4	2880360-2883463	567
VV05G10260	*	GSVIVT00020467001	<i>NCED</i>	5	11817325-11819052	575
VV10G02580	*	GSVIVT00028310001	<i>NCED</i>	10	3218097-3219371	424
VV11G02770		GSVIVT00016619001	unknown	11	2145852-2149875	438
VV13G12460	*	GSVIVT00028786001	<i>CCD1</i>	13	14371816-14380481	542
VV13G12530	*	GSVIVT00028793001	<i>CCD1</i>	13	14459328-14467975	542
VV16G00950		GSVIVT00013941001	<i>CCD4d</i>	16	720579-721262	227
VV16G01030	*	GSVIVT00013949001	<i>CCD4c</i>	16	792204-793964	586
VV17G04310		GSVIVT00017633001	unknown	17	4192339-4192880	107
VV19G09570	*	GSVIVT00000988001	<i>NCED</i>	19	10593695-10595524	609
VV15G01930	*	GSVIVT00019146001	<i>CCD7</i>	15	3669884-3674064	626
VV04G03390	*	GSVIVT00032423001	<i>CCD8</i>	4	2783048-2785780	563

* Indicates the putative genes used to generate the phylogenetic tree illustrated in Figure 1.

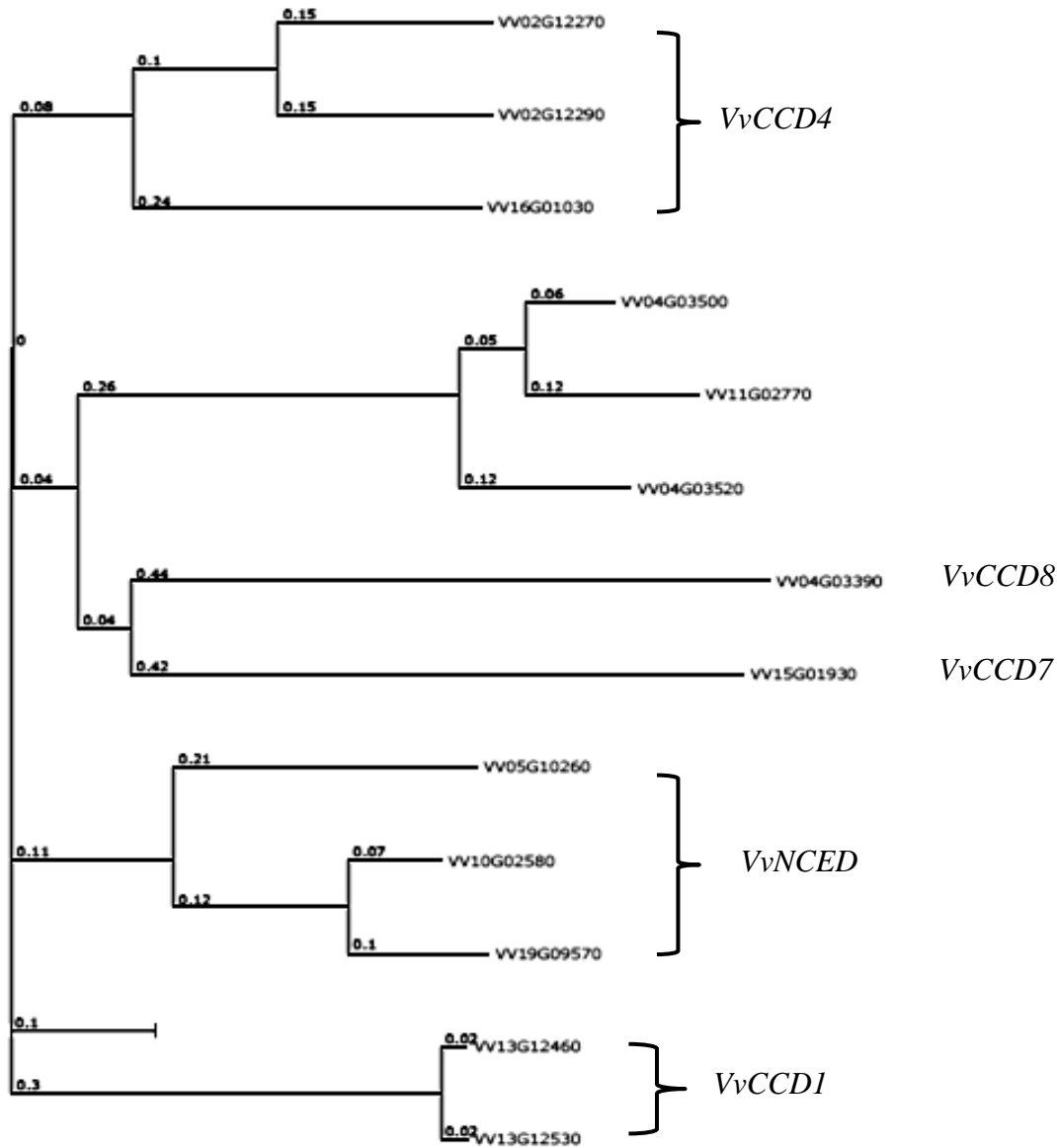


Figure 1. Phylogenetic tree of the VvCCD proteins based on amino acid sequence similarity. The putative genes marked with an asterisk in Table 5 were included in the phylogenetic tree. The predicted protein sequences were initially clustered using ClustalW (<http://www.clustal.org>). Accession numbers are listed in Table 5.

Full-length cDNAs of *CCD4* genes were isolated from *V. vinifera* to characterise the enzymatic activities of the encoded proteins. Gene fragments of *VvCCD4a* and *VvCCD4b* were first cloned using Reverse transcriptase PCR. Full length cDNAs of these genes were obtained using gene specific primers (Table 1). *VvCCD1*, *VvCCD4a* and *VvCCD4b* were successfully cloned in pTWIN1, a pRARE cassette added and co-transformed with the pAC-plasmids as described in Cunningham & Gantt, (2007). Despite numerous attempts using numerous cDNA pools, *VvCCD4c*, *VvCCD4d*, *VvCCD7* and *VvCCD8* could not be isolated. Moreover, the expression analysis (see section 3.3.2.) also confirmed that these genes were not expressed in any of the tissues tested, under the conditions used for this study.

3.3.2. Expression of grapevine CCDs encoding genes

The expression of *VvCCD1*, *VvCCD4a* and *VvCCD4b* was measured by Real-time PCR at the different stages of berry ripening and in various organs of *V. vinifera* (cv Pinotage). *VvCCD4c*, *VvCCD4d*, *VvCCD7* and *VvCCD8* expression was not detected in any of the cDNA pools tested (Table 6). Results were normalised to the expression of *EF1 α* (expression of this genes is considered constant during grape berry development according to Terrier et al. 2005; Reid et al. 2006; Guillaumie et al. 2011). The expression pattern of *VvCCD1*, *VvCCD4a* and *VvCCD4b* was similar in the ripening period, although *VvCCD4b* transcripts were more abundant than *VvCCD1* and *VvCCD4a*, with the latter having the lowest transcript abundance of the three. All three genes were up-regulated at véraison (Figure 2) with a strong induction in expression level evident for *VvCCD4b* in ripening berries (Figure 2). *VvCCD1* expression was found to be highest in leaf and in flower (respectively a four- and two-fold change in expression relative to green berry was observed as shown in Table 6). *VvCCD4a* expression, on the other hand, was highest in leaf and in flower (respectively a fourteen- and three-fold change in expression relative to green berry as shown in Table 6). A three-fold change in expression, relative to green berry was observed for *VvCCD4b* in leaf and flower (Table 6).

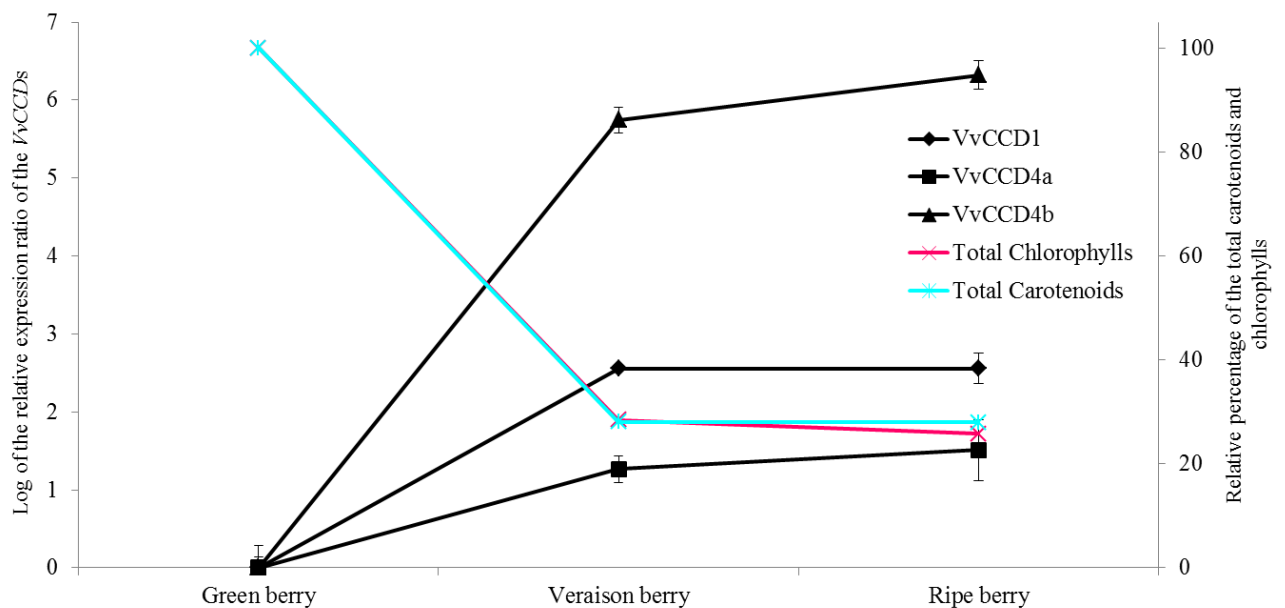


Figure 2. Real time PCR analysis *VvCCD1*, *VvCCD4a* and *VvCCD4b* gene expression in Pinotage leaf, flower and berry tissue. Data is expressed relative to the green berry stage and is normalised to *EF1 α* expression. The total carotenoid and chlorophyll content that was present in the Pinotage leaf, flower and berry tissue is represented on the secondary axis. Data is expressed relative to the green berry stage and is normalised to the internal standard β -apo-caroten-8-ol.

Table 6. Carotenoid and chlorophyll concentrations in Pinotage leaf, flower and during three stages of berry development and the relative expression of the VvCCD genes. Carotenoids and chlorophylls (in ng/g FW) were quantified using HPLC. Relative expression was determined using Real-time PCR and is expressed relative to the green berry developmental stage. “ND”, not detected. Data represents the average of n=3. Due to the high concentrations of carotenoid and chlorophylls in leaf and flower (relative to berry), these values were excluded from the bar graphs.

	Leaf	Flower	Green	Veraison	Ripe
Antheraxanthin	55470	8396	739	598	541
β-carotene	62687	15401	4228	1015	911
Chlorophyll a	868480	288956	69749	18403	16380
Chlorophyll b	289905	119338	32254	10615	9907
Lutein	97121	37838	8059	1965	1890
Lutein 5,6 epoxide	0	28	82	14	12
Neoxanthin	36192	13116	3454	828	842
Violaxanthin	67683	9356	2858	570	461
Zeaxanthin	97121	8856	0	323	298
Total Carotenoids	421232	95574	20967	5888	5867
Total Xanthophylls	353586	79189	16442	4815	4763
Total chlorophylls	1158385	408294	102003	29018	26287
VvCCD1	2.07	7.2	1	5.87	5.89
VvCCD4a	2.78	104.81	1	2.4	2.85
VvCCD4b	1.15	1.18	1	53.66	80
VvCCD4c	ND	ND	ND	ND	ND
VvCCD4d	ND	ND	ND	ND	ND
VvCCD7	ND	ND	ND	ND	ND
VvCCD8	ND	ND	ND	ND	ND

3.3.3. Carotenoid content in organs of *V. vinifera*

The carotenoid content in leaves, flowers, as well as during the different developmental and ripening stages of grapevine berries, was determined in the Pinotage cultivar. The concentrations (in ng/g FW) of the carotenoids and chlorophylls in the leaves, flowers and ripening berries are listed in table 6. As expected (Razungles et al. 1987), the total carotenoid content decreased as ripening progressed (Figure 2 & 3). The total chlorophyll content also decreased from the green to the ripe stage (Figure 2 & 3). There was a positive correlation between the chlorophylls and some of the individual carotenoids. The degradation pattern of the chlorophylls as ripening progressed was followed by the carotenoids lutein, β-carotene, neoxanthin and violaxanthin. Lutein was present as 36% of the total carotenoids, β-carotene at 18.7% of the total carotenoids and neoxanthin at 15.6% of the total carotenoids in the different stages of berry ripening. These results correspond with studies done on the carotenoid content in ripening berries (Gross, 1991; Razungles et al. 1993). The same pattern of degradation was present in flower material. Lutein

was present as 39.6% of the total carotenoids, β -carotene at 16.1% of the total carotenoids and neoxanthin at 13.7% of the total carotenoids. In leaves, zeaxanthin, lutein and β -carotene constituted 28.5%, 23.1% and 13.8% of the total carotenoids, respectively (Table 6).

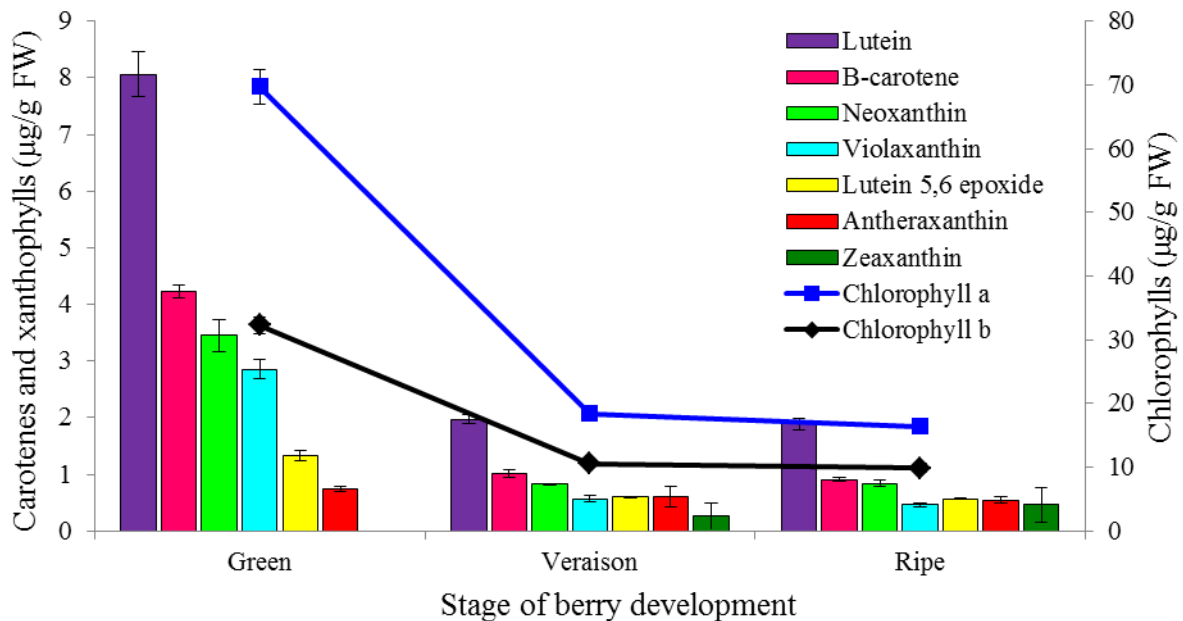


Figure 3. The individual carotenes, xanthophylls and chlorophylls present in the Pinotage berry tissue. Data is normalised to the internal standard β -apo-caroten-8-ol.

3.3.4. Functional characterisation of *VvCCD1*, *VvCCD4a* and *VvCCD4b*

To functionally characterise the *VvCCDs*, the headspace of the various carotenoid-accumulating *E. coli* strains expressing *VvCCD1*, *VvCCD4a* and *VvCCD4b* were analysed by HS-SPME-GC-MS analysis. Several optimisations were done to minimize the natural degradation of the carotenoids; to ensure that the apocarotenoid formation is predominantly due to the enzymatic cleavage by the *VvCCDs* and not due to oxidation or other non-enzymatic degradation. These optimisations are described in Addendum A. During the GC-MS sample preparation, at the point of CCD induction, samples were taken from culture to analyse the carotenoid content, therefore identifying and quantifying the substrates present for the enzyme reaction.

The concentrations of the apocarotenoids (products from the enzyme reaction) that were detected in the headspace of each of the carotenoid-producing *E. coli* strains are illustrated in Figure 4. An overview of the substrates analysed by UPLC analysis, the specific cleavage sites and the relative percentages of the apocarotenoids formed are presented in Table 7.

In theory, 6-methyl-5-hepten-2-one (MHO) and pseudoionone can be generated from the 5,6 (5',6) and 9,10 (9',10') double bond cleavage of lycopene. But in the headspace of the lycopene-producing *E. coli* strains which expressed *VvCCD1*, *VvCCD4a* and *VvCCD4b*, only MHO was detected (Figure 4). In the remaining carotenoid-accumulating *E. coli* strains, only 9,10 (9',10) double bond cleavage was noticed.

UPLC analysis (Addendum B to Chapter 3) confirmed that the majority of the carotenoid-accumulating *E. coli* strains produced the specific carotenoid defined by the pAC-plasmid, except the strains which theoretically produced ζ -carotene and ϵ -carotene. The UPLC analysis revealed that the ϵ -carotene-producing *E. coli* strain contained 70% ϵ -carotene and 30% lycopene at the point of protein induction. Both α -ionone and β -ionone was detected in the headspace as expected, with very low levels of pseudoionone, bordering on the levels of quantification for this compound, observed. In the ζ -carotene-producing *E. coli* strain, the UPLC analysis revealed that the strain contained 80% ζ -carotene and the remaining 20% was an unknown compound. In the headspace of the ζ -carotene-producing *E. coli* strains, only geranylacetone was detected, which was the expected apocarotenoid.

Table 7. A summary of the substrates used in the HS-SPME-GC-MS analysis, the UPLC analysis of the substrates present, the cleavage sites, as well as the relative percentages of the apocarotenoids formed. Expression of *VvCCD1*, *VvCCD4a* and *VvCCD4b* is relative to pTWIN1 and normalised to OD_{600nm} and the internal standard concentration (α -terpineol). Values indicated by * indicate a statistically significant difference ($p < 0.01$) between the pTWIN1 control and the respective *VvCCD*.

pAC-plasmid	Carotenoid (Substrate identified by UPLC analysis)	Cleavage activity	<i>VvCCD1</i>		<i>VvCCD4a</i>		<i>VvCCD4b</i>		Apocarotenoid (Products identified by GC-MS analysis)
pAC-PHYT	Phytoene (NA)	9,10 (9',10')	210.7	*	161.6	*	210.6	*	Geranylacetone
pAC-ZETA	ζ -carotene (80%) ^A	9,10 (9',10')	ND		ND		280.6	*	Geranylacetone
pAC-NEUR	Neurosporene (100%)	9,10 (9',10')	ND		172.8	*	166.3	*	Geranylacetone
pAC-LYC	Lycopene (100%)	5,6 (5',6')	168.0	*	192.3	*	198.9	*	MHO
pAC-EPSILON	ϵ -carotene (70%) ^B	9,10 (9',10')	835.3	*	474.4	*	651.2	*	α -ionone
		9,10 (9',10')	322.2	*	231.5	*	295.1	*	β -ionone
pAC-BETA	β -carotene (100%)	9,10 (9',10')	458.3	*	ND		ND		β -ionone
pAC-ZEAX	Zeaxanthin (100%)	9,10 (9',10')	ND		ND		ND		3-hydroxy- β -ionone

NA denotes that this carotenoid was not analysed by UPLC analysis.

^A denotes the percentage in brackets next to each carotenoid is an indication of the carotenoid content present in the specific carotenoid-accumulating *E. coli* strains, analysed by UPLC analysis. This indicated that 80% of the predicated carotenoid was ζ -carotene and 20% an unknown carotenoid.

^B denotes the percentage in brackets next to each carotenoid is an indication of the carotenoid content present in the specific carotenoid-accumulating *E. coli* strains, analysed by UPLC analysis. This indicated that 70% of the predicated carotenoid was ϵ -carotene and 30% was lycopene.

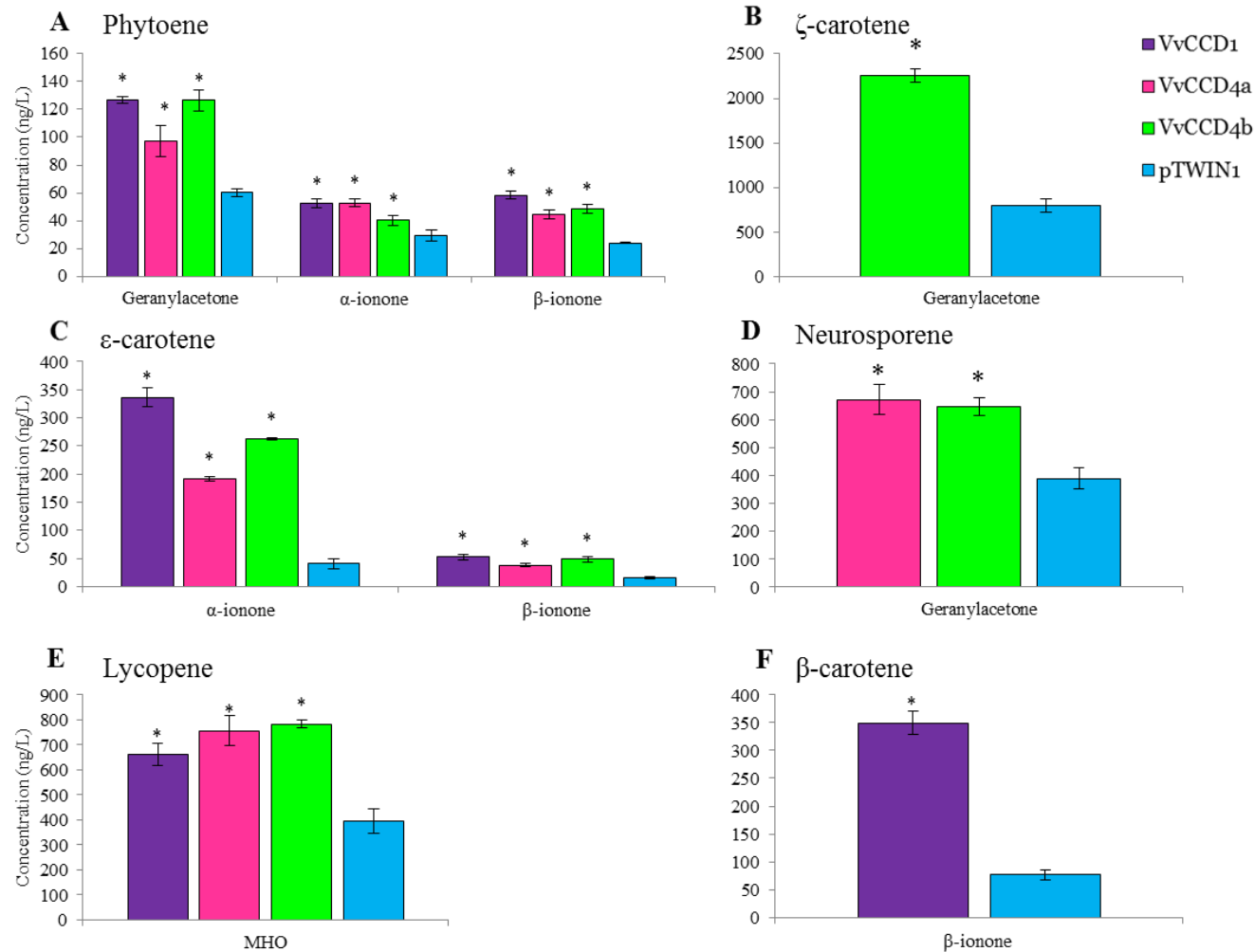


Figure 4. GC-MS analysis of the bacterial headspace. *Escherichia coli* strains were engineered to accumulate carotenoids and co-transformed with pTWIN1-VvCCD1-RARE (VvCCD1), pTWIN1-VvCCD4a-RARE (VvCCD4a), pTWIN1-VvCCD4b-RARE (VvCCD4b) and pTWIN1 (as negative control). Concentrations of apocarotenoids were expressed relative to the negative control and normalised to both the OD_{600nm} and the internal standard concentration (α -terpineol). Values indicated by an asterisk indicate a statistically significant difference ($p < 0.01$) ($n=3$). The carotenoid-accumulating strains contained pAC-PHYT (A), pAC-ZETA (B), pAC-EPSILON (C), pAC-NEUR (D), pAC-LYC (E), pAC-BETA (F).

3.4. Discussion

3.4.1. Isolation and expression analysis of grapevine CCD encoding genes

VvCCD4a and *VvCCD4b* were isolated from *V. vinifera* cv Pinotage and showed 73% similarity to each other. However, they have very different expression patterns in *V. vinifera*. The expression of *VvCCD4a* was predominantly in leaves with fewer transcripts present in flowers and the different stages of berry development. *VvCCD4b* transcripts were most abundant during berry development, with a strong induction in the ripening period. Moreover, there was a clear inverse relationship between *VvCCD4b* gene expression and carotenoid and chlorophyll content in the berries during development; when the total carotenoid and chlorophyll content was low, the expression levels of *VvCCD4b* was high. The *VvCCD4s* are present in the chloroplasts, since grapevine does not form chromoplasts (as in fruits such as tomato) (Ben-Amotz & Fishler, 1998); citrus (Pan et al. 2012); papaya (Skelton et al. 2006); pumpkin and winter squash (Paris, 1994). Differential expression patterns of *CCD4a* and *CCD4b* were also observed in *Chrysanthemum morifolium* (Huang et al. 2009b); maize (Vallabaneni et al. 2010) and citrus fruit (Pan et al. 2012). Huang et al. (2009b) suggested that *CCD4a* and *CCD4b* may have different substrates in plants, thus suggesting different biological functions.

In tomato, *SiCCD7* was strongly expressed in seed and root material (Liang et al. 2011) and *SiCCD8* transcript levels were highest in roots and stems (Kohlen et al. 2011). Roots proved to be the tissue that contained the highest levels of *ZmCCD7*, *ZmCCD8a* and *ZmCCD8b* (isolated from *Zea mays*) (Vallabhaneni et al. 2010). The expression of *ZmCCD7* in roots was consistent with the expression of orthologues in *Arabidopsis* (Booker et al. 2004; Sorefan et al. 2003; Auldridge et al. 2006b; Vogel et al. 2008); pea (Sorefan et al. 2003) and petunia (Snowden et al. 2005). Despite numerous attempts using numerous cDNA pools, including root cDNA, *VvCCD7* and *VvCCD8* could not be isolated from the grapevine material used in this study, and gene expression for these genes could also not be detected.

There are several factors which could have had an influence on *VvCCD7* and *VvCCD8* expression. The roots generated from the cuttings were not exposed to light prior to being harvested and stored for RNA extraction. Koltai, (2011) suggested that light is a positive regulator of SL biosynthesis. In tomato, the plants grown under light intensities above a certain threshold exhibited higher levels of SL in their roots and higher levels of *SiCCD7* transcription (Koltai, 2011). Under limiting phosphate conditions, plants respond in a number of different ways, all aimed at increasing the discovery of the rhizosphere for phosphate and the reduction in the investment of resources into the shoot. It has been demonstrated in several plant species, that

there is an up-regulation in SL production under phosphate starvation, therefore reducing the number of shoot branching events (Yoneyama et al. 2007; López-Ráez et al. 2008; Umehara et al. 2008; Jamil et al. 2011; Kohlen et al. 2011). López-Ráez et al. (2011) illustrated that AM symbiosis reduced SL biosynthesis by the host plant and that the reduction is dependent on the level of root colonisation. García-Garrido et al. (2009) suggested that a reduction in SL biosynthesis is a strategy by the plant to regulate mycorrhization, to avoid excessive mycorrhizal colonisation. The mechanism by which SL biosynthesis is reduced by AM symbiosis is unknown. Possible explanations include a direct effect on strigolactone production by mycorrhization or an indirect effect by the improvement of the plant's nutritional status (López-Ráez et al. 2011).

From more than 400 000 EST sequences generated to date (446,668 *Vitis vinifera* ESTs in the NCBI database (6 October 2012), there is no sequence data for either *CCD7* or *CCD8*. This does not mean that *VvCCD7* and *VvCCD8* are not expressed; it just implies that the tissue type or developmental stage that does express them has not been sequenced yet. Mutations of *CCD7* and *CCD8* have a noticeable phenotype in plants. Experiments using the synthetic SL, GR24, demonstrated that SLs (or a derived compound) could partially restore the wild-type branching phenotypes and bud outgrowth in *ccd7* and *ccd8* mutants of pea, rice and *Arabidopsis* (Gomez-Roldan et al. 2008; Umehara et al. 2008). Studies using Flouridone, an inhibitor of carotenoids biosynthesis, have illustrated that carotenoid biosynthesis is necessary for normal levels of SLs (Ito et al. 2010).

Towards the end of this study Fasoli et al. (2012) reported a genome-wide transcriptomic atlas of grapevine based on 54 tissue samples spanning most tissue types in grapevine. Young, green, vegetative tissues to mature, woody tissues; various organs at different developmental stages, including berries which had undergone post-harvesting withering and specialised tissues (pollen and senescent leaves) were represented. All together these samples expressed approximately 91% of the predicted grapevine genes. Expression data revealed *VvCCD7* to be present in very few (only 3/54) of the tissue samples, namely young inflorescence tissue (single flower in a compact group) and in the cluster rachis (at the fruit set and post fruit set stages). A rachis represents a highly branched structure, and it is feasible that its formation is controlled by the activation and inhibition of branching. This hyper-branched structure could be evidence of controlled branching and would be in line with SLs characterised role in the inhibition of shoot branching (reviewed by Dun et al. 2009; Domagalska & Leyser, 2011; Waters et al. 2012). No expression was detected for *VvCCD8* for any of the tissues tested (0/54). *CCD7* and *CCD8* are tightly transcriptionally regulated, as expected for phytohormone biosynthetic genes, but could

also be post-transcriptionally regulated in addition (Wang et al. 2011; Alder et al. 2012). Conversely, *VvCCD1* was present in all (54/54) and *VvCCD4a* and *-b* in most (40/54) of the tissue samples tested. Our data fits with the expression pattern observed for these genes by Fasoli et al (2012).

3.4.2. *VvCCD1, VvCCD4a and VvCCD4b encode functional CCDs able to cleave a range of substrates*

From *in vitro* studies, CCD1 and CCD4 enzymes cleave carotenoids at the same position 9,10 (9',10') double bonds and have a key role in the formation of β -ionone and other fruit and flower apocarotenoids (Rubio et al. 2008). Previous studies have illustrated that *VvCCD1* was able to cleave multiple carotenoid substrates *in vitro*: *VvCCD1* cleaved zeaxanthin and lutein to produce 3-hydroxy- β -ionone; but could not cleave β -carotene (Mathieu et al. 2005). Two additional substrates for *VvCCD1* were identified by Lashbrooke (2010), namely lycopene (producing MHO and pseudoionone) and β -carotene (producing β -ionone). This study confirmed the findings of Lashbrooke et al (2010) and identified two additional substrates for *VvCCD1*: phytoene and ϵ -carotene forming geranylacetone and α - and β -ionone respectively.

Although *VvCCD4s* have previously been identified, their functionality has not been demonstrated (Guillaumie et al. 2011; Young et al., 2012). This study revealed the 5,6 (5',6') and 9,10 (9',10') double bond cleavage of several substrates for both *VvCCD4s*. *VvCCD4a* cleaved phytoene, neurosporene, lycopene and ϵ -carotene, whereas *VvCCD4b* cleaved phytoene, ζ -carotene, neurosporene, lycopene and ϵ -carotene.

3.4.3. *What does the substrate specificities reveal about the possible in planta functions of VvCCD1 and VvCCD4*

The essential roles of carotenoids in photosynthesis, photomorphogenesis and plant development, suggest that their metabolism (biosynthesis and catabolism) is co-ordinately regulated with other major processes, including plastid biogenesis, flowering and fruit development (Fraser & Bramley, 2004). Specific regulatory mechanisms of carotenoid biosynthesis operate in photosynthetic tissue and in flowers and fruits (Lu & Li, 2008). In chloroplasts, carotenoids are involved in a number of functions crucial to photosynthesis such as light-harvesting, the reaction centre complexes and protecting the photosynthetic apparatus from photo-oxidation (Ahrazem et al. 2010; Zhu et al. 2010). Thus the maintenance of the optimal carotenoid composition and amount is crucial for the functioning of photosynthesis. The steady state levels of carotenoids are expected to be dependent on the storage capacity of plastids, but also on the balance between biosynthesis and degradation (Ruiz-Sola & Rodríguez-Concepción,

2012). Thus there is continuous turnover of carotenoids and chlorophyll in photosynthetic tissues.

In planta, CCD1 and CCD4 differ in the subcellular location, CCD1 being cytosolic (or on the outer membrane of the chloroplast) and CCD4 being plastidial. The presence of plastid target peptides and the confirmed plastid localisation of the CCD4 and CCD7 enzymes, allows these enzymes access to carotenoid substrates, illustrating their role in carotenoid maintenance and apocarotenoid synthesis (Rubio et al. 2008; Brandi et al. 2011). Since CCD1 enzymes do not have direct access to the carotenoids located in the plastids (Auldridge et al. 2006b; Rubio et al. 2008; Baldermann et al. 2010; Brandi et al. 2011), it is thought that CCD1 enzymes cleave C₂₇ intermediates that are exported from the chloroplast to the cytosol. During senescence, when the chloroplast membranes disintegrate, CCD1s potentially then have access to C₄₀ carotenoid substrates (Wise & Hooper, 2007). C₂₇ apocarotenoids have rarely been found in nature (Walter et al. 2010); perhaps due to the activity of CCD1s in the plant tissue given the fact that they are ubiquitously expressed, as Fasoli et al. (2012) illustrated. It is speculated that plant CCD1s also convert the plastid-released C₂₇ apocarotenoids that have arisen through the non-enzymatic oxidative cleavage processes (Ilg et al. 2010). Therefore, CCD1s are thought to not directly be involved in carotenoid maintenance in photosynthetic tissue.

The results from this study, as well as previous studies (Mathieu et al. 2005; Lashbrooke, 2010) revealed that VvCCD1 cleaved β -carotene and zeaxanthin, which are directly involved in photosynthesis. The carotenes (especially β -carotene) are enriched in the photosystem reaction centres, whereas the xanthophylls (zeaxanthin) are most abundant in the light-harvesting complexes. In chloroplasts the abundance of chlorophylls masks the colours of carotenoids. It is only when chlorophylls are degraded that the carotenoids become visible in leaves (Wise & Hooper, 2007). VvCCD1 would thus be able to cleave carotenoids that it would only be able to gain access to during senescence or when the plastid membrane is damaged. β -carotene; a carotenoid involved in photosynthesis, was present as 18.7% of the total carotenoids in the different stages of berry ripening and this compound, as well as zeaxanthin were both substrates for VvCCD1, but not for VvCCD4a or -b.

The results from this study revealed that VvCCD4a and -b could not cleave the carotenoids involved in photosynthesis (β -carotene and zeaxanthin). VvCCD4a and -b were however functional and cleaved several other carotenoids, namely phytoene, ζ -carotene, neurosporene, lycopene and ϵ -carotene. These carotenoids however do not occur at detectable levels in grapevine (as observed in this study in the tissue tested). Why do plants contain these compartmentalised enzymes that are transcriptionally regulated and functional, acting on specific

substrates which do not occur? That fact that these substrates could not be detected could be attributed to the VvCCD4s activity, causing only the specific carotenoids to be maintained (not cleaved). This suggests that the VvCCD4s are involved in carotenoid maintenance, as CCD4s are plastidic (Rubio et al. 2008; Brandi et al. 2011) and carotenoids are synthesised and present in the plastid as well (Howitt & Pogson, 2006). These results correspond with the suggestions from Brandi et al. (2011) that CCD4s are actually responsible for the maintenance of carotenoid turnover in photosynthetic organs and that CCD1s are involved catabolising carotenoids during senescence, as well as cleaving C₂₇ compounds, forming volatile apocarotenoids.

AtCCD4, *AtCCD1* and a number of carotenoid metabolic enzymes, from *A. thaliana* (Vidi et al. 2006; Ytterberg et al. 2006) and *CsCCD4* from *C. sativus* (Rubio et al. 2008) have been identified in the plastoglobule proteome. Lipid droplets called plastoglobules (PGs) are present in all tissues and plastid types, such as chloroplasts, chromoplasts and leucoplasts (Deruere et al. 1994; Pozueta-Romero et al. 1997; Kessler et al. 1999; Vidi et al. 2006; Ytterberg et al. 2006; reviewed by Piller et al. 2012). PGs are structures associated with protein-lipid membranes of thylakoids and are involved in the optimisation of photosynthesis, light acclimation and repair (Lundquist et al. 2012). PGs also protect the thylakoid membranes against oxidative stress (Brehelin & Kessler, 2008). During senescence plastoglobules increase in size and number, while the thylakoid membranes disintegrate and disappear (Gaude et al. 2007; Brehelin & Kessler, 2008). Similar to the identification of *AtCCD4*, *AtCCD1* and *CsCCD4* in the PG proteome, *VvCCD1*, *VvCCD4s* or *VvCCD7* may also be present in the PG proteome. This contributes towards the hypothesis that CCD1s might cleave C₄₀ compounds such as β-carotene and zeaxanthin during senescence. It would be interesting to investigate whether VvCCD8 could also possibly be present in the PG proteome, as this would correlate with the hierarchical clustering analysis reported by Fasoli et al. (2012) that grouped *VvCCD8* to the category genes that are expressed in leaves undergoing senescence.

3.5. Conclusion

This study has identified putative *VvCCD1*; *VvCCD4a*; *VvCCD4b*; *VvCCD7* and *VvCCD8* (*in silico*); isolated *VvCCD4a* and *VvCCD4b*; functionally characterised *VvCCD1*; *VvCCD4a* and *VvCCD4b* and determined the expression of *VvCCD1*; *VvCCD4a* and *VvCCD4b* in the different *V. vinifera* organs and stages of berry development. This study has also identified additional substrates for VvCCD1 *in vivo*, which has contributed to the understanding of possible roles of *VvCCD1* *in planta*. The identification of the substrate β-carotene for VvCCD1 *in vitro* contributed to the hypothesis that VvCCD1 cleaves C₂₇ apocarotenoids and C₄₀ compounds

during senescence, when photosynthesis is no longer a priority and β -carotene is no longer required.

Guillaumie et al. (2011) were unable to illustrate the functionality of VvCCD4a. This study revealed the 5,6 (5',6') and 9,10 (9',10') double bond cleavage of several substrates by both VvCCD4s. VvCCD4a and VvCCD4b could not cleave the carotenoids involved in photosynthesis, but cleaved several other carotenoids, namely phytoene, ζ -carotene, neurosporene, lycopene and ϵ -carotene. These results correspond with those of Brandi et al. (2011) and corroborate the suggestions that CCD4s are responsible for the maintenance of carotenoid turnover in photosynthetic organs.

Fasoli et al. (2012) revealed for the first time that VvCCD7 was expressed in two very distinct organs and no expression data was available for VvCCD8 for any of the 54 tissues sequenced. The fact that genomic copies of both VvCCD7 and VvCCD8 exist and the encoded enzymes are essential for normal plant functioning, lets one postulate that these genes are expressed and functional, but are under strict transcriptional regulation. These genes are possibly tightly regulated since the enzymes are involved in phytohormone biosynthesis (Wang et al. 2011; Alder et al. 2012). In the absence of protein and/or enzymatic data, it is impossible to speculate on the role(s) of VvCCD7 and VvCCD8 in grapevine. Although VvCCD7 and VvCCD8 could not be isolated and functionally characterised, testing different tissues (e.g. rachis) could lead towards the successful isolation of VvCCD7 and VvCCD8.

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Addendum A to Chapter 3

Optimisations for the determination of volatiles from bacterial headspace by GC-MS analysis

Experiments involving carotenoids (as well as apocarotenoids) can be very challenging, as carotenoids can naturally degrade (oxidation by non-specific enzymes (Carail & Caris-Veyrat, 2006) or by photochemical processes or other conditions causing oxidative stress (reviewed by Walter & Strack, 2011)) to form compounds which are of interest (apocarotenoids). This complicates assigning a function to an enzyme as so many factors contribute to the formation of apocarotenoids. This addendum contains a discussion of the factors tested to improve the analysis of apocarotenoids. Factors evaluated include: the bacterial expression system (e.g. rare codons, induction temperature and IPTG concentration), carotenoid production (e.g. minimising light exposure, the use of a carotenoid inhibitor and the concentration of the inhibitor used; co-factors and antioxidants), volatile apocarotenoid extraction (temperature of extraction, NaCl addition and the volume of culture) and GC analysis (internal standard and GC-MS settings).

To functionally characterise VvCCD1, VvCCD4a and VvCCD4b isolated from *Vitis vinifera*, the respective genes were cloned into pTWIN1 vector for expression in *Escherichia coli* as described in Chapter 3 (section 3.2.6). For substrates, the plasmids expressing VvCCD1, VvCCD4a and VvCCD4b were introduced into *E. coli* strains that accumulate the following carotenoids per strain: phytoene, ζ -carotene, neurosporene, lycopene, ϵ -carotene, β -carotene and zeaxanthin as described in Chapter 3 (section 3.2.7). The headspace was subsequently analysed by HS-SPME-GC-MS. Several factors were evaluated during sample preparation (bacterial cultures) as well as during the GC-MS analysis to facilitate maximum substrate (carotenoid) formation, optimal enzyme functioning and product (apocarotenoid) formation.

A 1. Optimisations of factors related to bacterial expression and carotenoid formation

A 1.1. Light and temperature

Initially the cultures were grown in the light at 37°C and continued to grow in the light at 37°C after the addition of the carotenoid inhibitor (DPA), prior to the protein induction step that proceeded in the dark at room temperature. Mathieu et al. (2005) decreased the temperature from 37°C to 18°C during protein production to overcome the formation of inclusion bodies.

Therefore samples were placed at room temperature during protein induction to facilitate the correct formation of the proteins. Additionally Ruther et al. (1997) grew bacterial cultures at 28°C and reported to provide the maximum yield of pigment. Therefore, to allow the maximum yield of carotenoids, as well as the correct protein formation, the cultures were kept at room temperature for the entire procedure. To prevent possible photo-degradation of carotenoids (Demmig-Adams et al. 1996; Demmig-Adams, 1998; reviewed in Walter & Strack, 2011), light was restricted during all the stages of the sample preparation and protein induction (Lashbrooke et al. 2010).

A 1.2. *pRARE cassette*

Several laboratories illustrated that expression yields of proteins whose genes contained rare codons could be dramatically improved when the cognate tRNA was increased within the host (Brinkmann et al. 1989; Siedel et al. 1992; Rosenberg et al. 1993). To improve protein expression, a pRARE cassette was integrated into the pTWIN1 expression vectors. The pRARE cassette encodes tRNA genes for all the “problematic” rarely used codons encoding Arg, Ile, Gly, Leu and Pro.

A 1.3. *Concentration of diphenylamine*

To use a biological system for the *de novo* production of substrates can be quite challenging. CCDs are problematic to express in bacteria as they tend to be insoluble. Another problem is that the carotenoid substrates are lipophilic and are therefore not soluble in an aqueous solution, therefore making the *in vitro* assays quite challenging. The bacterial cultures designed to produce the various carotenoids (as described by Cunningham & Gantt, 2007) were allowed to grow until the exponential growth phase, after which a carotenoid inhibitor diphenylamine (DPA) (Sigma) was added to prevent the synthesis of additional coloured carotenoids, as suggested by Cunningham & Gantt, (2007). Cunningham & Gantt, (2007) illustrated that 200 µM was the most effective concentration of DPA to use to prevent synthesis of additional carotenoids. It was shown that concentrations greater were inhibitory to the growth of the *E. coli*. Initially a concentration of 200 µM was added to each sample two hours prior to induction of protein (CCD) synthesis with IPTG (Roche), as suggested by Cunningham & Gantt, (2007), but alternative concentrations were tested as the DPA was building up on the SPME fibre and co-eluting with the internal standard (3-octanol) in the subsequent GC-MS analysis. Therefore lower concentrations of the DPA were tested (70 and 100 µM). No DPA was detected by the system at 70 µM, but the concentration was too low to inhibit carotenoid formation, therefore a concentration of 100 µM was selected. The co-elution problem was reduced but not eliminated. Since the DPA was crucial for the analysis, an alternative solution was to select an internal

standard (α -terpineol) that did not co-elute with DPA and continue to use a concentration of 100 μ M of DPA. The DPA did not interfere with the new internal standard (α -terpineol) as the retention times were different. A concentration of 100 μ M of DPA was used for the final protocol as it ensured inhibition, without the build-up and co-elution on the GC-MS system.

A 2. Optimisations of factors related to apocarotenoid extraction

A 2.1. The effect of flash-freezing on apocarotenoid extraction from bacterial samples

Carotenoids can be degraded enzymatically or via non-specific mechanisms such as (photo) chemical oxidation (reviewed by Walter & Strack, 2011). As an alternative to placing all the samples simultaneously on the GC-MS instrument, (to eliminate the formation of possible degradation products in the samples analysed later in the sequence) flash-freezing the samples were evaluated. Cultures of pTWIN1 and pTWIN1-VvCCD1 which had been co-transformed with pAC-BETA (see Table 2 in Chapter 3 for the description of the plasmids) were used to determine the effect that flash freezing had on the samples. Six identical samples of each strain were prepared. Triplicates of the samples were flash frozen after the 16 hours of IPTG induction and stored at -80°C . The triplicates of the fresh samples were placed immediately on the instrument. The individual frozen samples were removed 45 minutes prior analysis to allow thawing and then placed on the GC-MS instrument. Figure A1 illustrates that there was a 3-fold difference between the non-frozen cultures (fresh) and the cultures that were frozen. Freezing the samples resulted in a loss of the compound of interest (β -ionone) (the reason for this is not clear and was not investigated further). As a result, all the samples were kept fresh and were placed simultaneously on the GC-MS instrument in the final protocol.

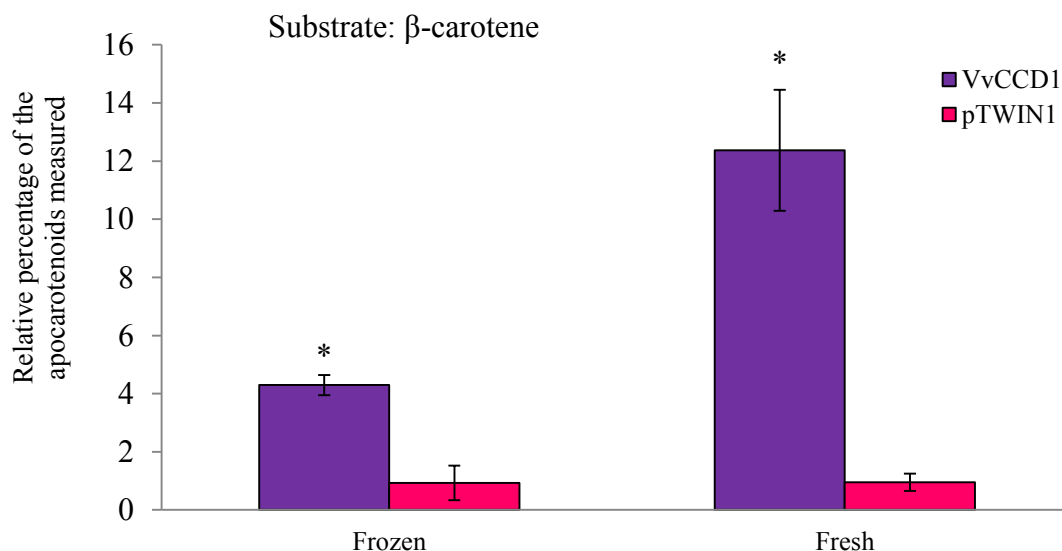


Figure A1. The relative percentages of β -ionone produced by the fresh (non-frozen) samples, versus the samples that were flash-frozen are shown. The percentage of the apocarotenoids produced by the bacterial cultures containing VvCCD1 is expressed relative to the percentage of the compound of interest produced by the bacterial cultures containing only pTWIN1 (control) and normalised to OD_{600nm} and the internal standard (3-octanol). ($P < 0.05$) (n=3)

A 2.2. *The effect of the addition of NaCl to the media (at 2.5 M NaCl) or resuspension in 5 M NaCl, on apocarotenoid extraction*

To determine whether harvesting the cells and resuspending in 5 M NaCl (Riedel-de Haen) or the addition of 2.5 M NaCl to the cultures after protein induction would stop the enzymatic reaction and prevent possible enzymatic degradation of the (apo)-carotenoids (not by CCD but by endogenous bacterial enzymes that can also affect carotenoids), cultures of pTWIN1 and pTWIN1-VvCCD1 which had been co-transformed with pAC-BETA were used.

To determine whether harvesting the cells and resuspending in 5 M NaCl would prevent possible degradation and stop the enzymatic reaction, six identical samples of each culture were prepared. Half of the samples of each culture were prepared according to the protocol described in Chapter 3 (section 3.2.8), whereas as the other half of the samples of each culture were not placed into the 20 mL GC vials after induction with IPTG. The cells of these samples were harvested after 16 to 20 hours of protein induction and resuspended in 5 mL of 5 M NaCl. These samples were then placed in the 20 mL GC vials and all the samples were placed on the GC-MS instrument simultaneously. Figure A2 illustrates that a significant amount of apocarotenoids were present within the media, which was discarded when the cells were resuspending in NaCl.

To test if the addition of 2.5 M NaCl to the cultures after protein induction would force the equilibrium of volatiles to the headspace, as well as prevent degradation, six identical samples of each culture were prepared. All of the samples of each culture were prepared according to the usual protocol. After 16 to 20 hours of protein induction, 2.5 M NaCl (Riedel-de Haen) was

added to half of the samples and all of the samples were placed on the GC-MS instrument simultaneously. Figure A3 illustrates that the addition of NaCl to the samples improved the extraction of the compound of interest (β -ionone). As a result, salt (2.5 M NaCl) was added to the samples after protein production in the final protocol, but the media was not discarded.

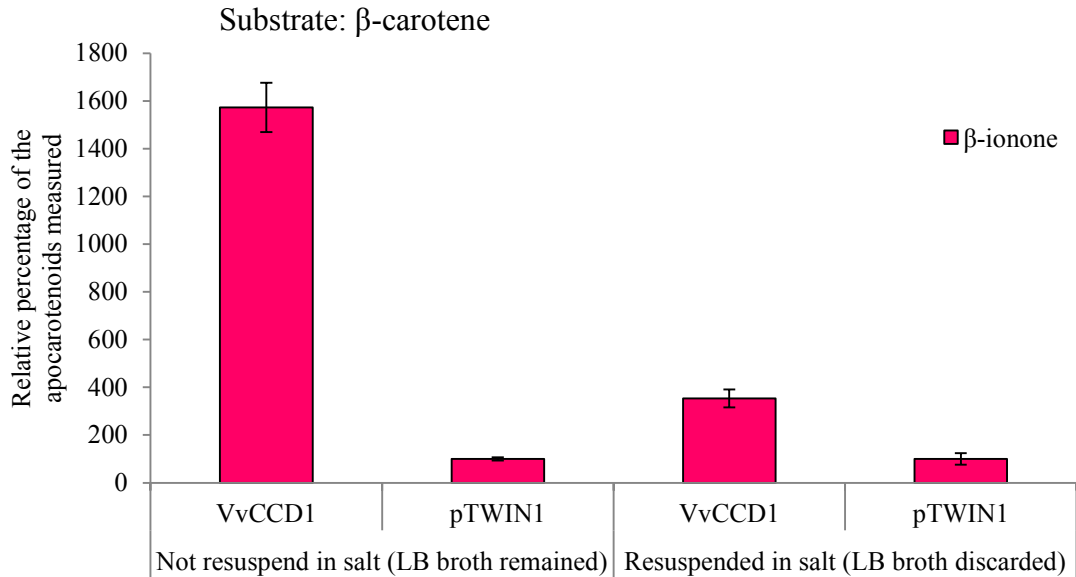


Figure A2. Relative percentages of β -ionone produced by the samples resuspended in 5 M NaCl and samples in Luria Burtani broth (no NaCl addition). The percentage of the apocarotenoids produced by the bacterial cultures containing VvCCD1 is expressed relative to the percentage of the compound of interest produced by the bacterial cultures containing only pTWIN1 (control) and normalised to OD_{600nm} and the internal standard (3-octanol). (n=3)

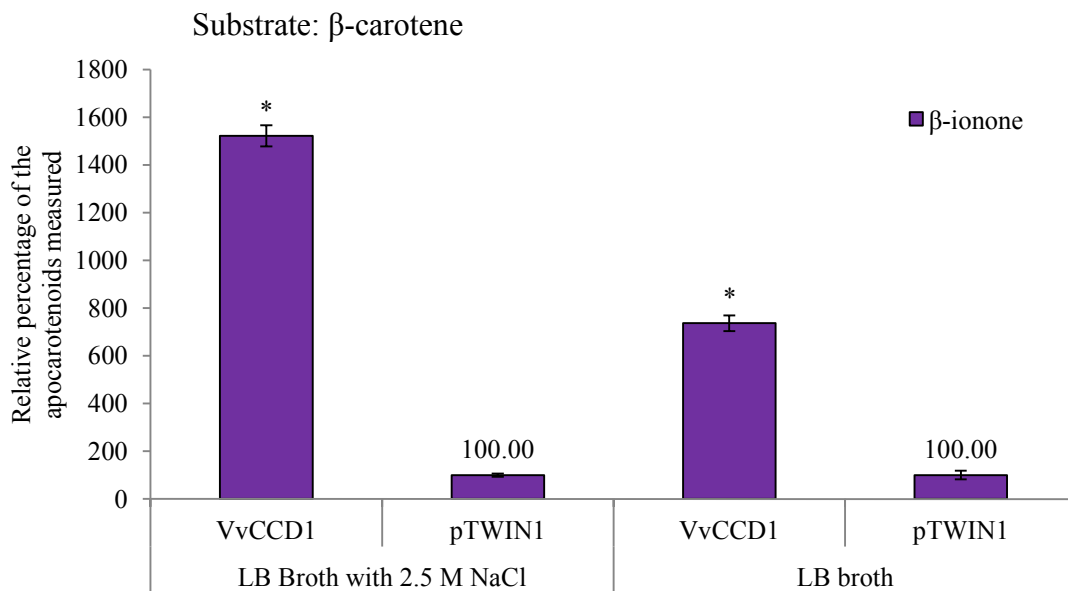


Figure A3. Relative percentages of β -ionone produced from the cultures with and without the addition of 2.5 M NaCl to the LB broth. The percentage of the apocarotenoids produced by the bacterial cultures containing VvCCD1 is expressed relative to the percentage of the compound of interest produced by the bacterial cultures containing only pTWIN1 (control) and normalised to OD_{600nm} and the internal standard (α -terpineol). (n=3) ($P < 0.05$)

A 2.3. Volume of cultures

The levels of the compounds of interest being detected and quantified were very low (ppt). Therefore the initial volume of culture was tested to see which would produce the maximum compound of interest. Cultures of pTWIN1 and pTWIN1-VvCCD1 which had been co-transformed with pAC-BETA were used and overnight cultures of each were prepared. The overnight cultures were used to inoculate four repeats (25 mL culture volume) of each culture to an OD_{600nm} of 0.1. Both cultures were prepared according to the protocol described in section 3.2.8 of Chapter 3. The cultures were separated into four sample volumes (6 mL, 12 mL, 24 mL and 48 mL) and the cells from each volume were harvested prior to protein induction with IPTG. The collected cells were resuspended in 6 mL of fresh media containing the appropriate antibiotics, IPTG and internal standard (α -terpineol). These samples were then placed in the 20 mL GC vials and incubated in the dark, gently shaking at room temperature for 16 to 20 hours. All the samples were placed simultaneously on the GC-MS instrument. Figure A4 illustrates that 24 mL of culture produced the highest concentration of the compound of interest (β -ionone). Therefore 24 mL size cultures were selected for the final protocol.

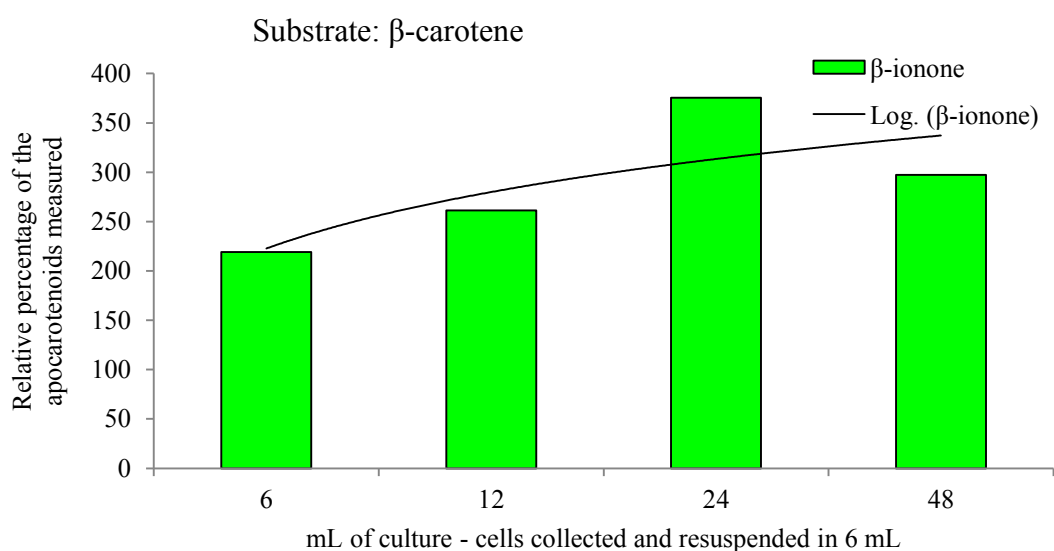


Figure A4. Relative percentages of β -ionone produced by the different initial volumes of culture. The percentage of the apocarotenoids produced by the bacterial cultures containing VvCCD1 is expressed relative to the percentage of the compound of interest produced by the bacterial cultures containing only pTWIN1 (control) (not represented in the graph) and normalised to OD_{600nm} and the internal standard (α -terpineol).

A 2.4. *The effect of the addition of a CCD co-factor and anti-oxidants (catalase and ascorbate) on apocarotenoid extraction from bacterial cultures*

Baldermann et al. (2010; 2012) illustrated the importance of the addition of the co-factor ferrous iron (Fe^{2+}) for enzyme functioning and therefore improved the *in vitro* enzyme assay as described in Fleischmann et al. (2002). After cultivation of the *E. coli* cells in the absence of the FeSO_4 , the isolated enzyme (OfCCD1, CCD1 isolated from *Osmanthus fragrans*) showed no activity due to the lack of FeSO_4 . Baldermann et al. (2012) added FeSO_4 (5 μM), catalase (200 U/mL) and ascorbate (6 mM) at the induction of CCD production. The FeSO_4 was added to improve the enzymatic cleavage of the OfCCD1, as was illustrated in Baldermann et al. (2010) and the anti-oxidants (ascorbate and catalase) were added to prevent chemical degradation of the carotenoids from (photo)-chemical oxidation (reviewed by Walter & Strack, 2011).

To determine whether the addition of FeSO_4 (5 μM), catalase (200 U/mL) and ascorbate (6 mM) as described by Baldermann et al. (2012), would improve the production of the compound of interest (geranylacetone) and reduce the background formation of apocarotenoids, cultures of pTWIN1 and pTWIN1-VvCCD4b which had been co-transformed with pAC-ZETA were used. Six identical samples of each culture were prepared. All of the samples of each culture were prepared according to the protocol described in Chapter 3 (section 3.2.8). At the point of protein induction, FeSO_4 , catalase and ascorbate were added to half of the samples. After 16 to 20 hours of protein induction, all of the samples were placed simultaneously on the GC-MS instrument. Figure A5 illustrates the 4-fold increase of geranylacetone in the cultures containing the additives relative to the control (pTWIN1), indicating that more of the compound of interest was produced. Figure A6 illustrates a much higher percentage ($\pm 500\%$) of the total additional compounds of interest produced by chemical degradation in the cultures not containing the additives, therefore also indicating that pTWIN1 (with additives) forms less “background” apocarotenoids via non-enzymatic degradation due to the presence of the anti-oxidants.

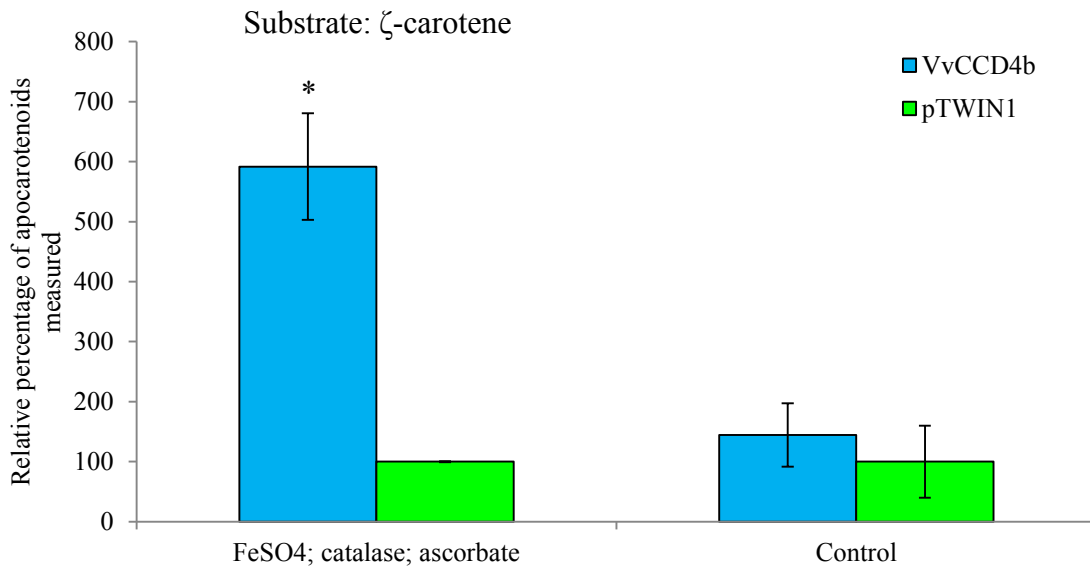


Figure A5. The relative percentages of geranylacetone produced by cultures with and without the additives (FeSO₄, catalase and ascorbate). The percentage of the apocarotenoids produced by the bacterial cultures containing VvCCD4b is expressed relative to the percentage of the compound of interest produced by the bacterial cultures containing only pTWIN1 (control) and normalised to OD_{600nm} and the internal standard (α -terpineol). ($P < 0.05$) (n=3)

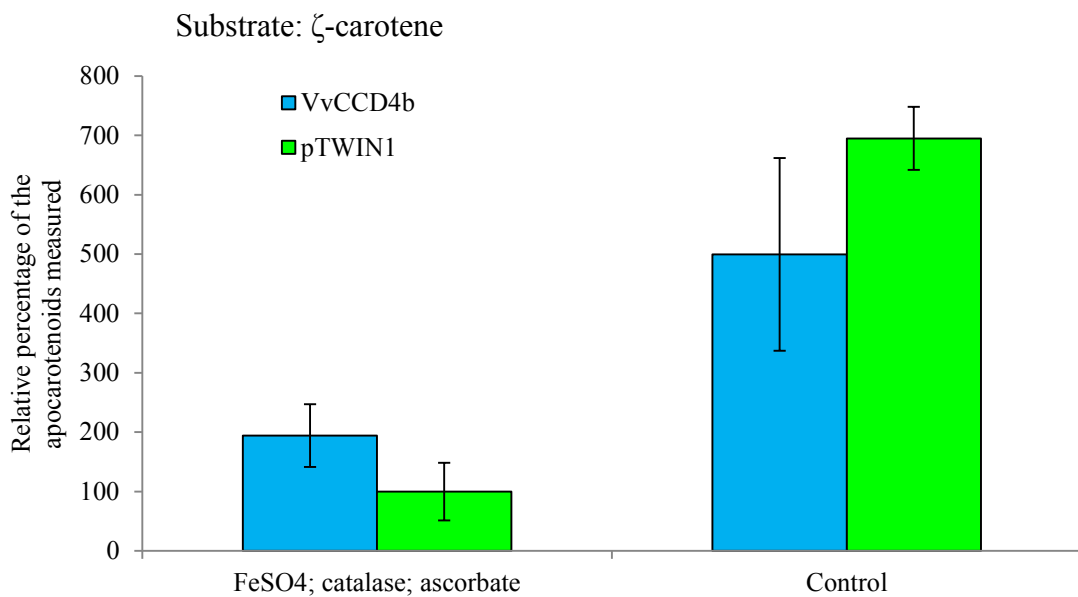


Figure A6. The relative percentages of the total additional apocarotenoids produced by cultures (not associated with CCD activity) with and without the additives (FeSO₄, catalase and ascorbate) are shown. The percentage of the total apocarotenoids produced by the bacterial cultures containing VvCCD4b (with and without the additives) and only pTWIN1 (without the additives) is expressed relative to the percentage of the total compounds of interest produced by the bacterial cultures containing only pTWIN1 with additives and is normalised to OD_{600nm} and the internal standard (α -terpineol). ($P < 0.05$) (n=3)

A 3. Optimisations of factors related to GC-MS analysis

A 3.1. Choice of extraction

HS-SPME analysis was selected to be the form of extraction.

A 3.2. Choice of fibre

The divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre (Supelco, Bellefonte, PA) was selected for the HS-SPME-GC-MS analysis. Alternative fibres were not evaluated, as this is considered the most suitable fibre for the extraction of apocarotenoids (Barros et al. 2012).

A 3.3. Extraction temperature

Lasshbrooke (2010) used an extraction temperature of 80°C, but the high temperature could result in the degradation of carotenoids (Ruther et al. 1997; Mathieu et al. 2005), therefore a lower temperature was evaluated. The extraction temperatures of 40°C and 80°C were assessed. The extraction temperature of 40°C gave better results (results not shown); therefore extraction temperature of 40°C was used in the final protocol.

A 3.4. Choice of internal standard and concentration

The initial internal standard used was 3-octanol (Sigma). To determine the concentration required, a concentration range in the same matrix as the samples was set up. An alternative internal standard was then selected as 3-octanol and diphenylamine (DPA) (a carotenoid inhibitor) were co-eluting at the same retention time (± 17 minutes). This made normalisation of compounds of interest to the internal standard impossible, as it was challenging to accurately integrate the internal standard. To improve this problem a range of concentrations of DPA were tested (section A 1.4), as well as resuspending the cultures in fresh media or 5 M NaCl (section A 2.2). The problem still continued, therefore an alternative internal standard was then selected, α -terpineol (Fluka, Buchs, Switzerland), as it eluted at a retention time of ± 21.35 minutes, that did not co-elute with the compounds of interest, or DPA. Therefore α -terpineol was used as the internal standard in the final protocol. To determine the concentration required, a concentration range in the same matrix as the samples was set up.

A 3.5. Calibration curves and quality controls

Concentrations ranging from parts per trillion (ppt) to parts per billion (ppb) of the authentic standards listed in Section 3.2.8 of Chapter 3 were used to determine the calibration curves. A quality control was analysed prior to the analysis of the first sample and another after the analysis of the last samples. The quality controls were also compared between experimental runs to give an indication of the condition of the fibre, as well as the running of the system. The

specific m/z fragments that were monitored for each apocarotenoid are listed in Section 3.2.8 of Chapter 3 and are illustrated in Figures A7 to A13, as well as the specific retention times that these compounds eluted.

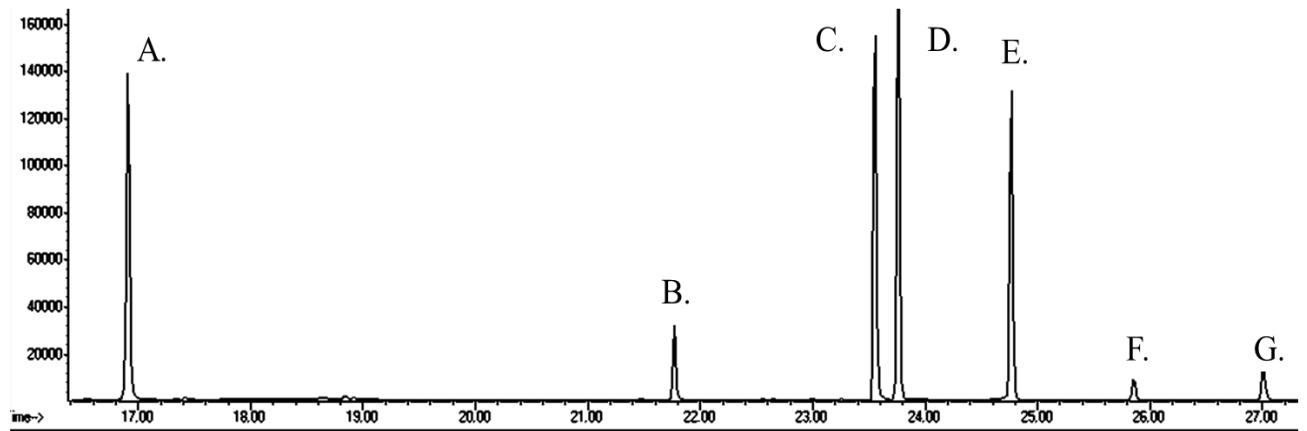


Figure A7. The retention times that the apocarotenoids which were monitored by HS-SPME-GC-MS analysis time eluted. (A) 6-methyl-5-hepten-2-one (MHO); (B) α -terpineol; (C) geranylacetone; (D) α -ionone; (E) β -ionone and (F) and (G) pseudoionone.

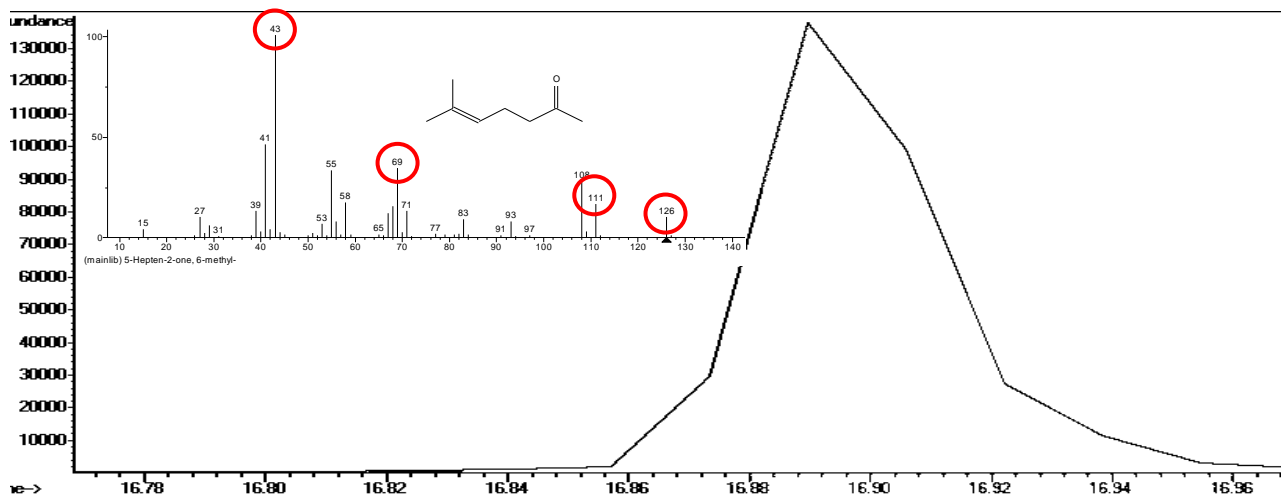


Figure A8. The retention time that MHO eluted is illustrated. The m/z fragments which were monitored are circled in red.

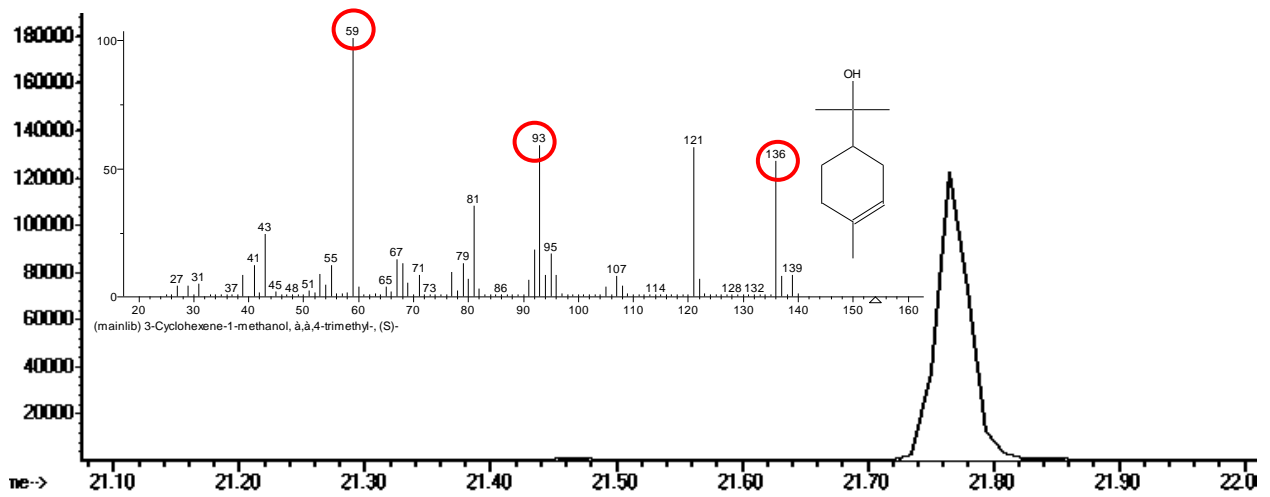


Figure A9. The retention time that α -terpineol eluted is illustrated. The m/z fragments which were monitored are circled in red.

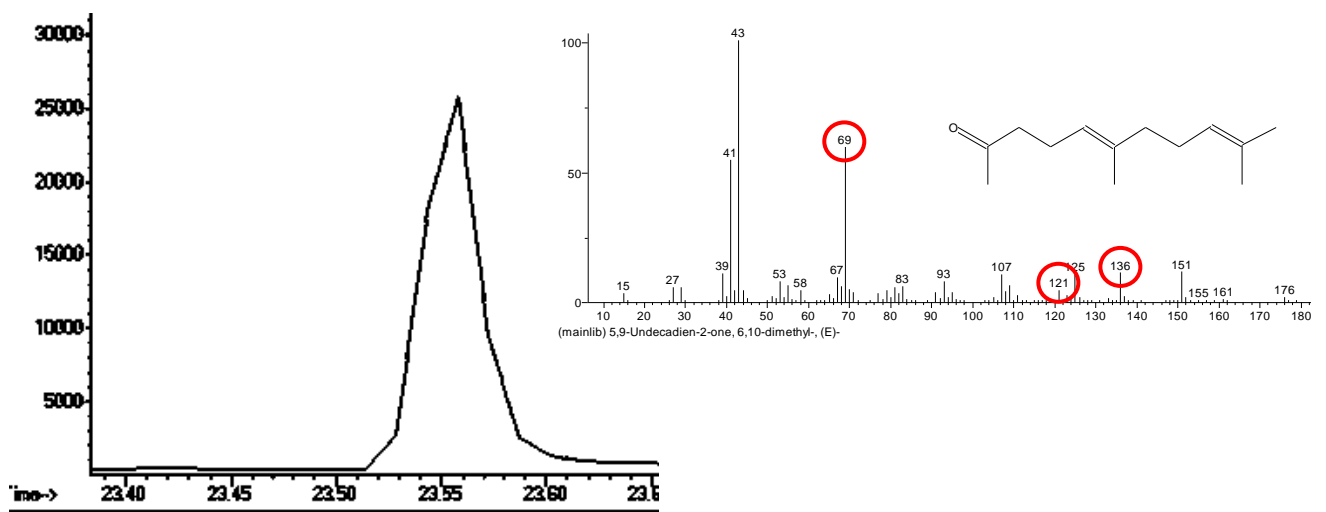


Figure A10. The retention time that geranylacetone eluted is illustrated. The m/z fragments which were monitored are circled in red.

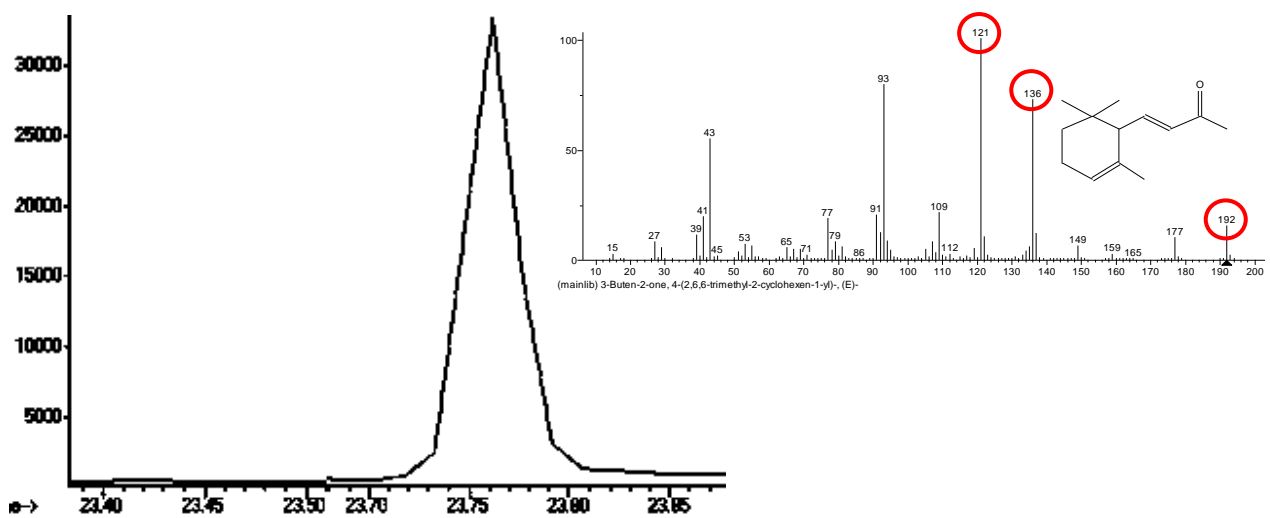


Figure A11. The retention time that α -ionone eluted is illustrated. The m/z fragments which were monitored are circled in red.

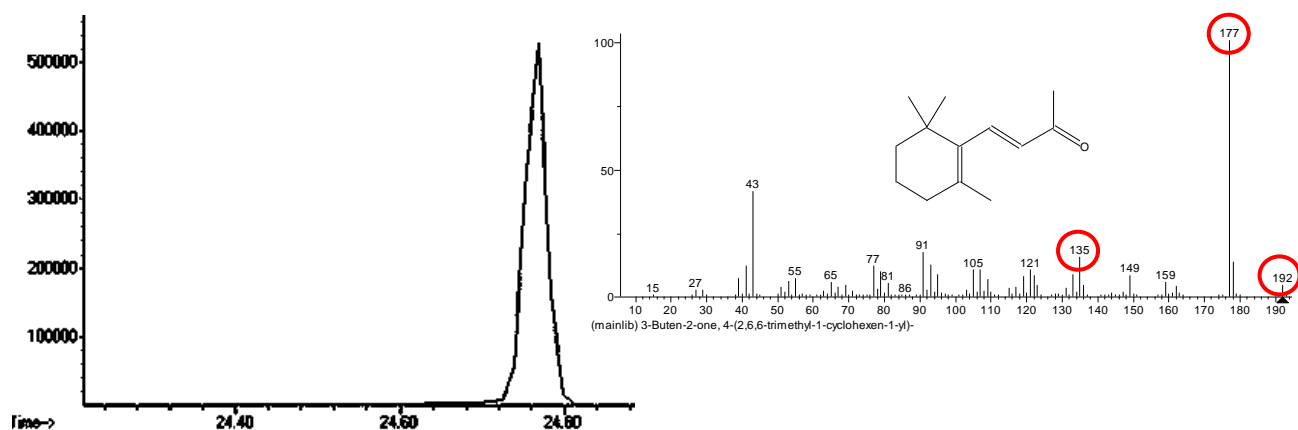


Figure A12. The retention time that β -ionone eluted is illustrated. The m/z fragments which were monitored are circled in red.

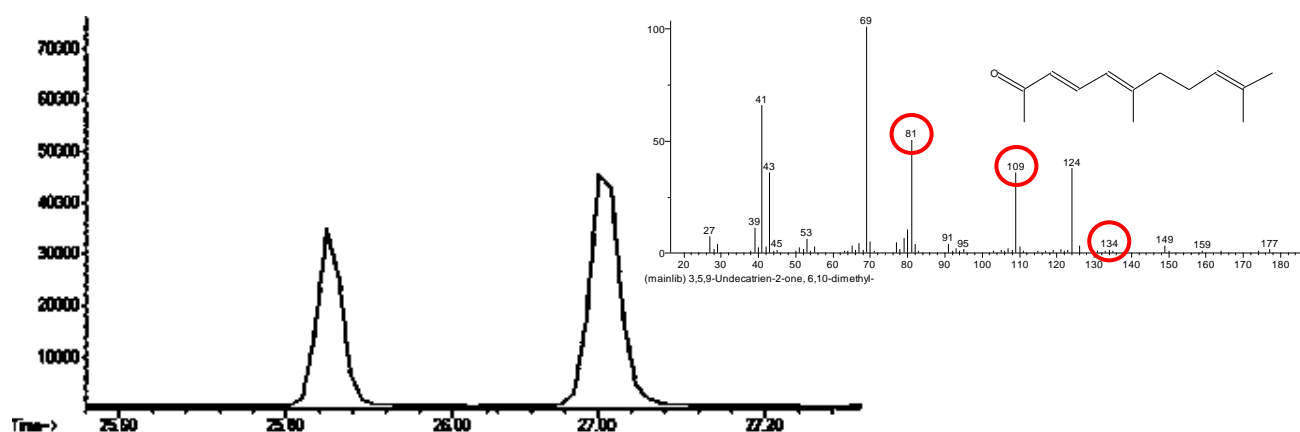


Figure A13. The retention time that pseudoionone eluted is illustrated. The m/z fragments which were monitored are circled in red.

A 4. Conclusion

The difficulties in analysing carotenoids and their degradation problems were identified and systematically addressed. Bacterial expression was optimised by the addition of the pRARE cassette, as well as growing and preparing the samples at room temperature. The carotenoids production/formation was optimised by the addition of DPA. As well as protecting the carotenoids from the influence of light, the bacterial enzymes, as well as oxidation by the addition of the anti-oxidants, the addition of 2.5 M NaCl and preparing the samples in the dark. Therefore non-enzymatic degradation of the carotenoids was minimised to give us a better resolution to analyse the formation of the compounds of interest (apocarotenoids). The VvCCD expression was improved by the addition of the CCD co-factor Fe^{2+} . The implementations of these optimisations resulted in a reliable, repeatable protocol for the preparation of the bacterial cultures as samples for HS-SPME-GC-MS analysis of apocarotenoids.

A 5. References

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Addendum B to Chapter 3

This Addendum contains all the data collected by UPLC analysis of the carotenoids present in the bacterial cultures at the point of CCD induction as described in section 3.2.10 of Chapter 3 and discussed in section 3.3.4 of Chapter 3, as well the details regarding the UPLC analysis. Table B1 contains the percentages of the predicted carotenoids formed in bacterial cultures. Figure B1 and Table B2 contains the flow rates and elution compositions during the UPLC analysis.

Table B1. The percentage of the predicted carotenoid substrates identified from the bacterial cultures. ND denotes not determined.

pAC-plasmid	Carotenoid (Substrate identified by UPLC analysis)	Phytoene	ζ-carotene	Neurosporene	Lycopene	ε-carotene	β-carotene	Zeaxanthin	Unknown
pAC-PHYT	Phytoene (ND)	ND	ND	ND	ND	ND	ND	ND	ND
pAC-ZETA	ζ-carotene	0.00	80.00	0.00	0.00	0.00	0.00	0.00	20.00
pAC-NEUR	Neurosporene	0.00	0.00	100.00	0.00	0.00	0.00	0.00	0.00
pAC-LYC	Lycopene	0.00	0.00	0.00	100.00	0.00	0.00	0.00	0.00
pAC-EPSILON	ε-carotene	0.00	0.00	0.00	70.00	30.00	0.00	0.00	0.00
pAC-BETA	β-carotene	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00
pAC-ZEAX	Zeaxanthin	0.00	0.00	0.00	0.00	0.00	0.00	100.00	0.00

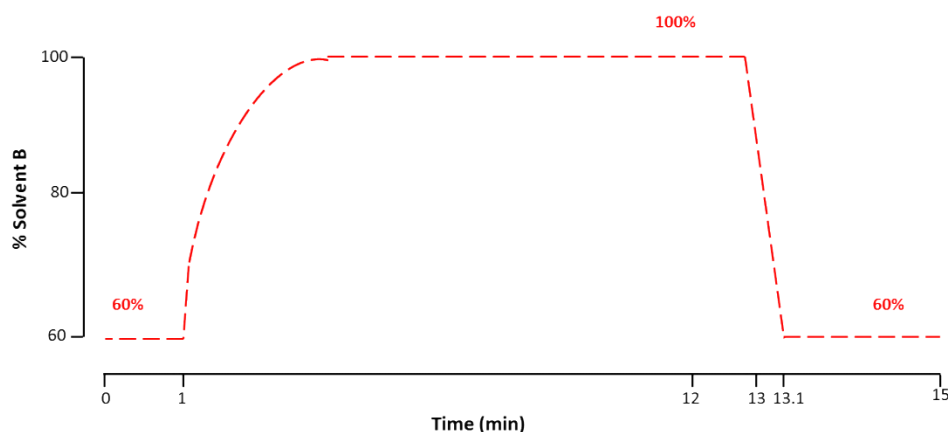


Figure B1. Eluent composition is depicted. The percentage of solvent B at each time point as mentioned in Table B2 is represented.

Table B2. Events, time laps, flow rates and eluent composition for the UPLC analysis

Ramping Events	Time (minutes)	Flow rate (mL/min)	% solvent system		Gradient curve
			A	B	
1	0	0.3	40	60	
2	1	0.3	40	60	6
3	12	0.5	0.2	99.8	3
4	13	0.3	0	100	6
5	13.1	0.3	40	60	6
6	15	0.3	40	60	6

Chapter 4

General discussion and conclusions

4.1. General discussion and conclusions

Plant carotenoid cleavage dioxygenases (CCDs) catalyse the oxidative cleavage of a number of carotenoid and apocarotenoid substrates, resulting in the formation of apocarotenoids that perform several biological roles plants, including hormones, pollinator attractants and flavour and aroma compounds (Huang et al. 2009). Several apocarotenoids are commonly extracted and used as flavourants and colourants in the food and cosmetic industry (Huang et al. 2009). Plant carotenoid biosynthesis occurs in plastids where they are bound in complexes with chlorophylls in the plastidic membranes (Demmig-Adams & Adams, 1996). Carotenoids have different roles, depending on the type of plastid they are synthesized and present in.

The CCD family of plants typically consists of CCD1s, CCD4s, CCD7s and CCD8s, in addition to the NCEDs. CCD1s have been shown to catabolise a wide variety of all-*trans*- and 9-*cis*-carotenoids, resulting in the formation of important apocarotenoid volatiles (e.g. β -ionone, β -cyclocitral, geranylacetone and pseudoionone) in the fruits and flowers of several plant species (Simkin et al. 2004; Auldridge et al. 2006b). After the NCEDs, which are involved in the biosynthesis of the plant hormone ABA (Schwartz et al. 1997), CCD1 orthologues are the best studied CCD, due to their involvement in the formation of C₁₃ apocarotenoids that contribute to the characteristic scents and aromas of flowers, fruits and wines (Baumes et al. 2002; Mendes-Pinto, 2009).

Despite this, several aspects of CCD functioning and also their *in planta* roles are still unclear and under active investigation. The aim of this work was therefore to focus on the grapevine CCDs: To perform expression analysis of the family in various grape organs and; to identify, isolate and functionally characterise the CCD family members from grapevine. The characterisation of the isolated CCDs particularly focused on proving that they encode functional CCDs, by evaluating their ability to cleave various carotenoid substrates. This latter step required a rigorous optimisation of a heterologous expression system in *Escherichia coli* to effectively produce (and measure) both the carotenoid substrates (through UPLC analysis) and the products of the CCD cleavage reactions (through HS-SPME-GC-MS analysis). The results obtained in this study and their impact in the field will be briefly highlighted below:

Previous studies have illustrated that the grapevine VvCCD1 was able to cleave multiple carotenoid substrates *in vitro*. Mathieu et al. (2005) identified a putative CCD1 from *V. vinifera* L. cv Shiraz (VvCCD1). To investigate whether VvCCD1 encoded a functional CCD, the co-expression of VvCCD1 in an *E. coli* strain which accumulated zeaxanthin was performed. When a comparison was made between the negative control and the strains containing VvCCD1, a lack

of colour in the colonies containing VvCCD1 was observed. To test the CCD activity, VvCCD1 was recombinantly produced in *E. coli* which was grown in liquid medium and the supernatant that was sonicated from the *E. coli* cells was used, with zeaxanthin as the substrate. GC-MS analysis revealed the presence of the predicted product, 3-hydroxy- β -ionone, which was not observed in the control. The assay was repeated with lutein as substrate, as lutein is one of the major carotenoids of grape berries. The same results were generated as with zeaxanthin as substrate, but no cleavage products were detected with β -carotene as substrate. It was therefore concluded that VvCCD1 was functional with 9,10 (9',10') cleavage activity. Lashbrooke (2010) also investigated the substrate specificity of a VvCCD1 isolated from *V. vinifera* L. Pinotage. The VvCCD1 was co-transformed in *E. coli* strains capable of accumulating phytoene, lycopene and β -carotene and the enzyme activity was monitored by measuring the formation of volatile apocarotenoid via HS-SPME-GC-MS analysis of the bacterial cultures. The expected apocarotenoids, MHO and pseudoionone (from lycopene) and β -ionone (from β -carotene) were formed. An additional cleavage activity at the 5,6 (5',6') double bond position of lycopene was also observed, confirming that VvCCD1 has 5,6 (5',6') and 9,10 (9',10') cleavage activity. In the current study a similar approach was used as by Lashbrooke (2010), but several optimisations were made to improve on the *in vivo* assay performed by Lashbrooke (2010) (as outlined in Chapter 3 and particularly Addendums A and B to Chapter 3 of this thesis). The optimised assay provided profiling and quantification data to confirm the carotenoid substrates and their levels at the point of CCD production, in addition to an optimised system to prevent non-enzymatic cleavage of the substrates and the correct co-factors to optimise CCD functioning. This system confirmed the previously known substrates and cleavage products for VvCCD1 and also identified phytoene and ϵ -carotene as additional substrates for VvCCD1.

Considering the substrates cleaved by VvCCD1, it raises the question how and when these enzymes function in the plant body. Given their ubiquitous expression patterns, as well as their cytosolic localisation, CCD1s potentially only have access to the C₄₀ carotenoids when the integrity of the photosynthetic/plastidic membranes are compromised during oxidative stress or during senescence when the plastid membranes start disintegrating (Wise & Hooper, 2007). Therefore the identification of the substrates β -carotene and zeaxanthin for VvCCD1 *in vitro*, suggests that VvCCD1 would only be able to cleave these C₄₀ compounds during senescence (Wise & Hooper, 2007), when photosynthesis is no longer a priority and β -carotene and zeaxanthin are no longer required. During berry growth, particularly towards the ripening stage, membranes become leaky and evidence of cell death had been found in (e.g. Shiraz berries) (Lang & Düring, 1991). Carotenoid-derived aroma compounds contribute to this cultivar's

distinct varietal character (Ristic et al. 2010). The expression data from this study and several others (Mathieu et al. 2005, 2007; Lashbrooke, 2010) has revealed an up-regulation of *VvCCD1* transcripts during berry ripening, which is when *VvCCD1* would have access to C_{40} carotenoids, thus releasing important aroma compounds. Possible *in planta* substrates for *VvCCD1* could also be C_{27} apocarotenoids which are generated from either enzymatic cleavage (via *CCD4* and/or *CCD7*) (Walter et al. 2010) or non-enzymatically (via e.g. photo-oxidation) (Ilg et al. 2010).

Although *in vitro* studies have shown that *CCD1* and *CCD4* enzymes cleave carotenoids at the same 9,10 (9',10') double bond positions, their substrate specificities differ, as was observed in this study. Previous studies were unable to functionally characterise the grapevine *CCD4* enzyme, *VvCCD4a* (Guillaumie et al. 2011), but this study revealed the 5,6 (5',6') and 9,10 (9',10') double bond cleavage of several substrates for both *VvCCD4a* and *VvCCD4b* (isolated in this study). Interestingly, *VvCCD4a* and *VvCCD4b* could not cleave the carotenoids predominantly involved in photosynthesis (i.e. β -carotene and zeaxanthin), which *VvCCD1* could cleave. *VvCCD4s* cleaved several other carotenoids, including phytoene, ζ -carotene, neurosporene, lycopene and ϵ -carotene to form geranylacetone, MHO, α - and β -ionone. It is known, and our study confirmed that these carotenoids do not occur at detectable levels in grapevine berries. The expression data from this study revealed *VvCCD4a* to be most prominent in flowers and a very strong induction in expression level of *VvCCD4b*, more than *VvCCD1* and *VvCCD4a*, was observed in the ripe berry tissue. Why does grapevine contain enzymes that are transcriptionally regulated and functional, acting on substrates that do not occur at detectable levels? It is possible that the *in planta* function of *VvCCD4a* is the maintenance of carotenoid turnover in leaves, flowers and berries. This would primary generates a C_{27} - and a C_{13} -apocarotenoid. The C_{27} -apocarotenoid could subsequently be transported from the plastid to the cytosol where it would be cleaved by *VvCCD1* to generate an additional C_{13} -apocarotenoid and a C_{14} -dialdehyde (Schwartz et al. 2001). These aspects; the possible localisation of the *CCDs* in the plastoglobules, as well as their general *in vivo* functions, present very interesting subjects for further studies.

Although orthologues of *VvCCD7* and *VvCCD8* could be found in the grapevine genome, the isolation was unsuccessful and no expression for *VvCCD7* and *VvCCD8* could be detected in any of the tissues or developmental stages tested in this study. However, Fasoli et al. (2012) revealed expression for *VvCCD7* in specialised tissue, namely in young inflorescence tissue and in the rachis. Fasoli et al. (2012) also could not detect *VvCCD8* expression in any of the 54 tissues and developmental stages analysed. Therefore, the expression data from this study corresponds to the data generated by Fasoli et al. (2012).

4.2. Conclusion and future prospects

In conclusion, the aims of the study were achieved and have added to the knowledge of plant CCDs. In this study, the grapevine CCD4s (VvCCD4a and VvCCD4b) were isolated and functionally characterised, contributing to their possible roles *in planta*, as maintaining carotenoid turnover in photosynthetic organs. Additional *in vitro* substrates were identified for VvCCD1, which supports the hypothesis that *in planta*, CCD1 cleaves C₂₇ apocarotenoids that is generated from either enzymatic cleavage (via CCD4 and/or CCD7) or photo-oxidation or the cleavage of C₄₀ carotenoids during senescence or when the membranes are “leaky”, generating volatile compounds, therefore contributing towards aroma in grapes and wine. Although VvCCD7 and VvCCD8 could not be isolated and functionally characterised, the use of specialised tissue in future, could lead towards the successful isolation of VvCCD7 and VvCCD8. Due to the floral and fruity characteristics that apocarotenoids contribute to wine, understanding the roles of carotenoids in photosynthesis, photo-protection and aroma-precursor production is crucial. Therefore this study has contributed to understanding of these roles and the enzymes (CCDs) which are responsible for the formation of the carotenoid-derived aroma compounds in grapes and wine.

4.3. References

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