# GENETIC DISSECTION OF A CANDIDATE GENE CONTROLLING FLOWER COLOR IN BRASSICA (FAST PLANT)

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Master of Science

(Plant Breeding)

by

[DIVYASHREE COIMBATORE NAGESWARAN]

[FEBRUARY 2016]

**ABSTRACT**

Carotenoids and apocarotenoids are essential for biological functions in plants, animals and humans. Different apocarotenoids are produced from cleavage of carotenoids at specific bond positions by the family of carotenoid cleavage dioxygenases (CCDs). The plant CCD4 enzymes have been reported to be expressed in flower tissues and preferentially cleave linear carotenoids. We hypothesized that CCD4 may be the gene that controls the flower color phenotype (white versus yellow) in Brassica (fast plant). Our results indicated that white flower color is dominant over yellow in petals of fast plants and is controlled by a single gene. qRT-PCR analysis showed much high expression of *CCD4b* in white flower compared to yellow flowers. Nucleotide sequences of *CCD4a* and *CCD4b* from fast plants were 99% identical and the genes are intron-less. The predicted amino acid sequences of CCD4 contained ~600 aa with chloroplast signal peptide in their N-terminus as well as several conserved histidine residues. Functional complementation of *CCD4a* and *CCD4b* in *E.coli* revealed significant β-carotene degradation and β-ionone release from cell extracts expressing both white and yellow CCD4b. Partial or low β-carotene cleavage was observed in *E.coli* harboring the *CCD4a* constructs. However, the carotenoid level and β-ionone release could not explain the petal color phenotype when analyzed through HPLC and GC-MS. Further experiments on molecular genetics (e.g. linkage analysis) may conclude the biological role of *CCD4* in controlling petal color.

## BIOGRAPHICAL SKETCH

Being a science lover and a scientific researcher has been my passion since my childhood days. I was born in Neyveli (INDIA) on 14th November 1989 and spent my school education at Madras. I had an excellent ambience and exposure during my school days towards academics, sports, literary and public speaking skills. I was one of the top students at 10th and 12th grade, a State level player in the game "Tennikoit", an orator at interschool level and a trained classical singer until present. I was an active participator in science exhibitions from 9th till 12th grade conducted at school and state levels, which introduced me to wide range of topics of great interest. My special focus towards agricultural sciences was embedded in me through inspiration from my grandfather, an agriculturist. Following his footprints, I joined B.Tech (Horticulture) at Tamilnadu Agricultural University (TNAU) in the year 2007 and graduated in 2011. As horticulture is all about understanding the science and production of fruits, vegetables, spices, plantation, medicinal & aromatic crops, it gave me new insights to deeply focus on exploring genetics, breeding and biotechnology that are key elements for crop improvement. So, I started M.Sc. (Biotechnology) at Tamilnadu Agricultural University in the year 2011 and then attained a place at esteemed Cornell University in 2012 through RATAN TATA FELLOWSHIP to pursue M.S. (Plant breeding) under a Dual degree program devised by TNAU-CORNELL partnership. I was very much privileged working in an international platform, which molded me to achieve higher standards in the course of my career. I returned back to India in 2013 with the wholesome experience, worked on my M.Sc. thesis research at TNAU and graduated in July, 2014.

[I dedicate this work to my beloved family members and respectful teachers]

**ACKNOWLEDGMENTS**

It is my privilege to express my sincere and heartfelt gratitude to my supervisor Dr. Li Li**,** Adjunct Professor at the Department of Plant breeding & Genetics and USDA-ARS scientist for her atmost guidance, help, motivation, attention, suggestions and wonderful supervision during the course of my research.

I express my esteem and profound sense of gratitude to my committee member Dr. Martha Mutschler, Professor at the Department of Plant breeding & Genetics for her valuable feedbacks during my first annual committee report presentation, which immensely helped me improve data interpretation skills in research to greater levels.

My special word of thanks to Devajit (visiting scientist) for his preliminary contribution to the project. I sincerely thank my beloved senior lab mate Dr. Xiangjun Zhou (Postdoc) who was an amazing mentor throughout my project. I thank my dear colleagues Dr. Hui Yan (Postdoc), Juinxiang (PhD student) and other lab members for their due guidance, help and support. I also express my gratitude to Dr. Joyce Van Eck at Boyce Thompson Institute for guiding me in Brassica transformation. I thank Karen, Melody and Dr. Donna Gibson from USDA-ARS, Robert Holley's Center for being very kind, caring and a helping hand during my obstacles.

I take this opportunity to thank RATAN TATA foundation and Tamilnadu Agricultural University (India) for providing me funding support for a year from 2012 to 2013. I wholeheartedly thank Department of Plant breeding & Genetics at Cornell University for providing me admission and for making my dream come true. I specially thank Mary Ellen Niederhofer, GFA and Janett for being very kind, caring, patient throughout in helping me out regarding all the graduate student affairs.

I express loads of thanks to my parents, grandparents, brother and my fiancé for their eternal love, affection, moral support and sacrifice in all aspects in order to cherish the moment of my victory in life.

Finally, I thank Almighty who made my dream a reality.

*Divyashree C Nageswaran*

**TABLE OF CONTENTS**

## LIST OF FIGURES

## LIST OF TABLES

**CHAPTER** 1

# INTRODUCTION

## 1.1. Carotenoids

Carotenoids belong to the class of $C_{40}$ isoprenoids that represent orange, red and yellow pigments widely distributed in nature. These indispensable plant pigments are synthesized and sequestered in the plastids (Cunningham and Gantt, 1998; Hirschberg, 2001; Howitt and Pogson, 2006; Taylor and Ramsay, 2005). More than 700 naturally occurring carotenoid compounds have been identified (Britton *et al*., 2004). Carotenoids accumulate largely in the chromoplasts of various flowers and fruits.

They form the accessory pigments and act as protection centres from excessive light *via* thermal energy dissipation in the photosystem assembly. The abundant xanthophylls in the photosynthetic plant tissues form the major components of light harvesting complexes. The xanthophyll pigments violaxanthin, lutein, zeaxanthin and antheraxanthin also act as photoprotective compounds that minimize photo-oxidative damage *via* non-photochemical quenching of singlet oxygen, thus limiting membrane damage in plants, algae and cyanobacteria (Niyogi, 1999; Muller *et al*., 2001; Demmig-Adams and Adams, 2002; Dellapenna and Pogson, 2006; Bailey and Grossman, 2008). Hence, carotenoids participate in various essential processes like photosynthesis, respiration, photoprotection, photomorphogenesis, non-photochemical quenching, lipid peroxidation and other regulatory processes (Dellapenna and Pogson, 2006; Cazzonelli *et al*., 2010).

### 1.1.1. Structure and Biological function

Carotenoids have polyene chains containing conjugated double bonds, which influence their chemical properties (El-Agamey *et al*., 2004). Carotenoids act as precursors for vitamin A biosynthesis. Vitamin A (all-*trans*-retinol) is an essential nutrient in mammalian diets that

occurs in the form of retinoids (von Lintig, 2010; Bai *et al*., 2011). Retinol is required for vision, maintenance, mucus secretion and reproduction. Each form is characterized by a retinyl group which consists of an unmodified β-ionone ring and an isoprenoid side chain.
The retinaldehyde (retinal) is used for the production of rhodopsin in the eyes as well as maintenance of epithelial and immune cells. The acidic form of vitamin A derivative called "retinoic acid" is a morphogen in development (Bai *et al*., 2011). The vitamin A activity is basically derived from both plant and animal sources. The retinyl esters present in animal meat and dairy sources are converted into retinol in the small intestine and further converted into retinal or retinoic acid. Human beings synthesize retinal in the intestinal mucosa enzymatically by β-carotene 15, 15′-monooxygenase from pro-vitamin A carotenoids to yield retinol (Bai *et al*., 2011; Mayne, 1996; von Lintig, 2010).

Plants are the rich sources of pro-vitamin A carotenoids which are generally present in dark green, yellow, and orange colored fruits and vegetables such as oranges, broccoli, spinach, carrots, squash, sweet potatoes and pumpkins (Harrison, 2005). The pro-vitamin A carotenoids such as α-carotene, β-carotene, γ-carotene, β-cryptoxanthin are converted into visual pigments in humans and β-carotene is the most efficient precursor owing to its two retinyl groups (Farre *et al*., 2010; von Lintig, 2010; Bai *et al*., 2011). In addition to β-carotene, other carotenoids such as lutein, zeaxanthin and β-cryptoxanthin have protective functions against cancer, stroke, macular degeneration, cataracts, and ageing (Mayne, 1996; Krinsky *et al*., 2003; Fraser and Bramley, 2004).

### 1.2. Carotenoid biosynthesis in plants

Carotenoids are organic tetraterprenoids composed of eight condensed $C_5$ isoprenoid precursors that generate a $C_{40}$ linear backbone. In plants, carotenoids are derived from two isoprene forms isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP)

through a condensation reaction occurring *de novo* within plastids (Chappell, 1995). The precursors are predominantly derived from plastid-localized methylerythritol-4-phosphate (MEP)/ mevalonate-independent pathway although the same precursors are produced by the cytosol localized mevalonic acid pathway (MVA; Rodriguez-Concepcion and Boronat, 2002; Rodriguez-Concepcion, 2006). The compounds pyruvate and glyceraldehyde-3-Phosphate act as initial substrates for the carotenoids derived from plastid-localized MEP pathway (Cazzonelli and Pogson, 2010). In plants like Arabidopsis, common precursors of isoprenoid synthesis are exchanged between cytosol and the plastid in the presence of light especially during seedling development (Rodriguez-Concepcion *et al*., 2004).

The entire biosynthesis pathway is shown in Figure 1. The first steps in the MEP pathway are catalyzed by deoxyxylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). The production of IPP (isopentenyl diphosphate) and DMAPP (dimethylallyl diphosphate) is catalyzed by the activity of 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR) and isopentenyl diphosphate isomerase (IPPI), respectively (Cazzonelli and Pogson, 2010; Bai *et al*., 2011). The subsequent steps involve the condensation of three IPP molecules with one DMAPP molecule that leads to the production of $C_{20}$ intermediate geranyl-geranyl diphosphate (GGPP) catalysed by the enzyme GGPP synthase (GGPPS). The process of condensation of two molecules of GGPP by phytoene synthase (PSY) yields the first carotenoid, phytoene. A series of four desaturation reactions are catalysed by the enzymes phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS). The first desaturation product is 9,15,9′-tri-cis-ζ-carotene which is isomerized by light exposure and/or ζ-carotene isomerase (Z-ISO) to produce 9,9′-di-cis-ζ-carotene, a substrate of ZDS and 7,9,9'-tri-cis-neurosporene. The final product of all the desaturation reactions is converted to all-*trans*-lycopene by the enzyme carotenoid isomerase (CRTISO; Isaacson *et al*., 2002; 2004; Chen *et al*., 2010; Li *et al*., 2007; 2010).

The cyclization of lycopene (ψ, ψ-carotene) is a key branch point in the pathway of carotenoid biosynthesis. There are two cyclic end groups found in higher plant carotenoids namely the β- and ε- rings. The carotenoids with two β rings are very unique and two ε rings are equally a rarity, unlike those with one β and one ε ring which are very common. Lycopene serves as a substrate for the enzymes lycopene β-cyclase (LYC-β) and lycopene ε-cyclase (LYC-ε). The ε-cyclase produces the monocyclic δ-carotene (ε, ψ-carotene) when added with one ring. The cyclization at both ends of the symmetrical and linear lycopene forms β-carotene (β, β-carotene). When β and ε cyclases combine, they convert lycopene to α-carotene (β,ε-carotene) possessing one β and one ε ring (Cunningham *et al*., 1996; Cunningham and Gantt, 2001).

The complex downstream carotenoids are synthesized by the process of hydroxylation catalysed by the non-heme β-carotene hydroxylases (CHY- β/CHY1, CHY2) and carotene ε-hydroxylases (CYP97A, CYP97C), which result in the formation of zeaxanthin from the β-carotene branch and lutein from the α-carotene branch. The xanthophyll compounds downstream of both α-carotene and β-carotene branch do not possess pro-vitamin A activity although zeaxanthin synthesis involves the production of an intermediate product, β-cryptoxanthin, which possesses pro-vitamin A activity (Tian *et al*., 2003; Quinlan *et al*., 2007; Diretto *et al*.,2007b; Bai *et al*., 2011; Giuliano, 2014). Zeaxanthin is first converted to antheraxanthin and then to violaxanthin by the addition of an epoxide group with enzyme zeaxanthin epoxidase (ZEP) which catalyses the two epoxidation reactions (Marin *et al*., 1996). Interconversion of violaxanthin to zeaxanthin takes place rapidly in the presence of violaxanthin de-epoxidase (VDE) under high light stress conditions (Pfundel and Bilger, 1994; Demmig-Adams and Adams, 1996; Cuttriss *et al*., 2011). Conversion of antheraxanthin and violaxanthin to neoxanthin is carried out by the enzyme neoxanthin synthase (NXS), whose genes have been identified in plant species for its activity (Al-Babili *et al*., 2000; Bouvier *et al*., 2000;

Cazzonelli and Pogson, 2010). The pathway downstream leads to the cleavage of 9-*cis*-violaxanthin and 9-*cis*-neoxanthin by nine-*cis*-epoxycarotenoid dioxygenase (NCED) and produce an intermediate xanthoxin, which is further modified to abscisic acid (ABA; Schwartz *et al*., 1997; Tan *et al*., 1997; Bai *et al*., 2011). Various other apocarotenoids (bixin, crocin, α-ionone, β-ionone, β-cyclocitral, pseudoionone, geranylacetone, etc.) are generated by the activity of carotenoid cleavage dioxygenases (CCDs), which utilize specific carotenoids as substrates (Bouvier *et al*., 2005; Auldridge *et al*., 2006a; Ohmiya, 2009; Cazzonelli and Pogson, 2010; Rubio-Moraga *et al*., 2014a).

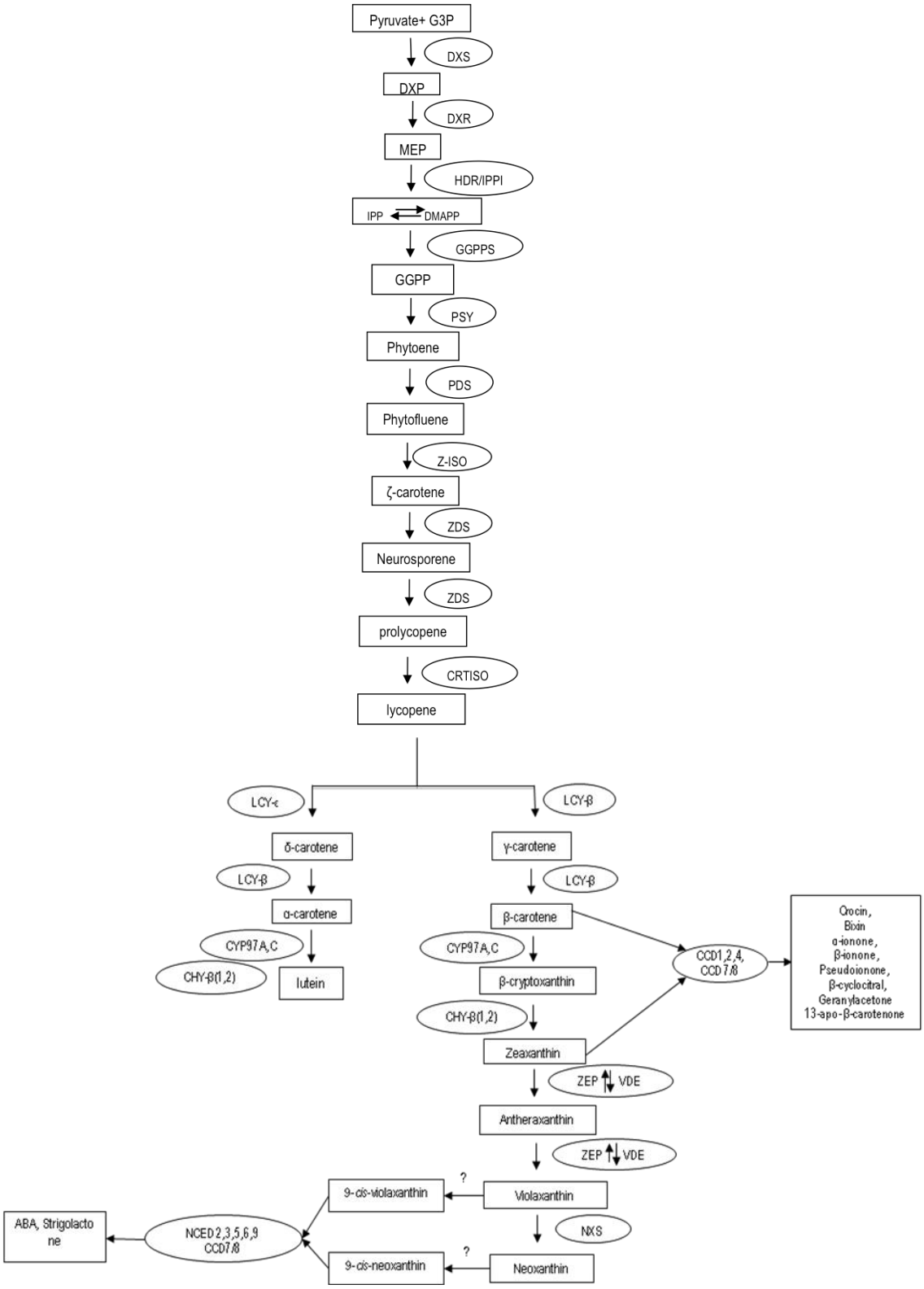



**Figure 1: Carotenoid biosynthetic pathway**

### 1.3. Metabolic engineering of carotenoids in food crops

Metabolic engineering of key carotenoid genes to enhance the levels of nutritionally important carotenoids such as β-carotene in food crops has been termed "biofortification" (Giuliano, 2014). People in developed countries have sufficient dietary diversity owing to their rich economic status and social awareness towards diet requirements. Micronutrient malnutrition (MNM termed "hidden hunger") is a serious health issue in developing nations as the dietary requirements are not met owing to poverty and high consumption of carbohydrate rich foods (Hirschi, 2009). Vitamin A deficiency is globally widespread where around 250,000-500,000 pre-school children become blind and about two-thirds die within months of severity. Approximately, 20 million pregnant women are vitamin A deficient especially in the developing countries (UNICEF, 2010; http://www.harvestplus.org/content/vitamin).
The Recommended Dietary Reference Intake (DRI) of vitamin A for males is 900 Retinol Amount Equivalents (RAE), 700 RAE for females, 770 RAE for pregnant females and 400-500 RAE for children, where 1 RAE is equivalent to 1 µg of retinol, 2 µg of β-carotene in oil and 12 µg of β-carotene in food (IOM, 2001). Hence, the global challenge is to combat Vitamin A deficiency through biofortification in staple food crops utilizing effective approaches to alleviate poverty, hunger and malnutrition.

Staple food crops such as rice, maize, cassava and potato have been modified through transgenic or classical breeding that elevates β-carotene levels (Bai *et al*., 2011; Giuliano, 2014). Conventional breeding tools utilize natural genetic variation for the target locus and undergo selection for the best genotype from the available germplasm. For example, genetic variation in maize *Lyc-ε* locus provides a wide range of carotenoid profiles and β-carotene content in the elite lines shows maximum of 13.6 µg/g DW (Harjes *et al*., 2008). Genetic engineering overcomes the limitations of traditional breeding through introduction of key carotenoid genes even from

diverse species, which have remarkably enhanced pro-vitamin A content in food crops. Recent studies document high accumulation of β-carotene up to 59.32 µg/g DW in transgenic multivitamin corn (Naqvi *et al*., 2009).

Phytoene synthase (PSY) is the most important enzyme acting as the rate limiting factor towards production of downstream carotenoids in crops like maize, wheat and cassava (Wong *et al*., 2004; Palaisa *et al*., 2004; Li *et al*., 2008; Welsch *et al*., 2010). The maize *y1* (yellow) locus was linked to *Psy1* gene (major QTL) that provides wide phenotypic variation in carotenoid levels ranging from 6.6-27.0% (Wong *et al*., 2004; Li *et al*., 2008). The first generation Golden Rice yielded 1.6 µg/g of β-carotene in the rice endosperm by stable overexpression of three major carotenoid genes of phytoene synthase (*Psy*) from daffodil, phytoene desaturase (*CrtI*) from *Erwinia uredovora* and lycopene β-cyclase from *Narcissus pseudonarcissus* (*Lyc-β*; Ye *et al*., 2000). The β-carotene levels were increased up to 31 µg/g DW in the II generation of Golden Rice following the replacement of *Psy* with maize *Psy* gene (Paine *et al*., 2005). In addition to cereal crops, crops like potato and tomato have been extensively utilized for implementing various metabolic engineering approaches (Giuliano, 2014; Fraser and Bramley, 2009). Overexpression of the bacterial *CrtB* encoding phytoene synthase gene led to significant accumulation of total carotenoids and β-carotene (11µg/g DW) in transgenic potato tubers (Ducreux *et al*., 2005). Similarly, overexpression of three bacterial mini-pathway genes (*CrtB*, *CrtI*, and *CrtY*) under tuber specific and constitutive promoters resulted in the development of golden potatoes with enhanced total carotenoids and β-carotene content up to 114 and 47 µg/g DW, respectively (Diretto *et al*., 2007a). In the same way, *Psy-1* overexpression from tomato increased β-carotene content to 1.5 folds whereas overexpression of *CrtI* from *Erwinia uredovora* in tomato enhanced β-carotene levels up to 3 folds (Fraser *et al*., 2007; Romer *et al*., 2000).

Lycopene ε-cyclase (LYCE) and β-carotene hydroxylases (CHY) are other key enzymes which play a pivotal role in accumulation of β-carotene and total carotenoids in crops like maize and potato (Harjes *et al*., 2008; Diretto *et al*., 2006). Tuber-specific downregulation of endogenous *Lyc-ε* directed the flux towards β-carotene branch thus increasing total carotenoid content by 2.5-fold and β-carotene levels by 14-fold in potato tubers (Diretto *et al*., 2006). Silencing of *chy-β* led to higher levels of total carotenoids and β-carotene in potato and sweet potato tubers (Diretto *et al*., 2007b; Kim *et al*., 2011). Similarly, silencing of potato *Zep* increased zeaxanthin levels and also total carotenoid content to 60.8 µg/g DW in tubers (Romer *et al*.,2002) whereas overexpression of *Lyc-β* and *Chy-β* from Arabidopsis enhanced accumulation of β-carotene, β-cryptoxanthin and zeaxanthin in tomato (Rosati *et al*., 2000; D'Ambrosio *et al*., 2004; Dharmapuri *et al*., 2002). Other dietary carotenoids such as astaxanthin possess tremendous importance in the food, cosmetic and pharmaceutical industries today due to their beneficial effects on human health. Natural astaxanthin is a potent antioxidant as well as a feed additive for fisheries (Huang *et al*., 2013; Giuliano, 2014). Metabolic engineering of two carotenogenic genes from algal species led to high yield of astaxanthin in tomato fruits up to 16.1 mg/g and a 16-fold increase in total carotenoids (Huang *et al*., 2013).

Although manipulating key genes through transgenics leads to the production of pro-vitamin A carotenoids, the desired carotenoid levels in food crops are yet to be achieved. Carotenoids accumulate largely in the chromoplasts which produce novel sequestering structures for its deposition (Howitt and Pogson, 2006; Li and Van Eck, 2007). A metabolic sink for carotenoid accumulation in food crops is controlled by a novel gene mutation termed the *Or* gene, which encodes a DnaJ cysteine rich domain containing protein (Li *et al*., 2001; Lu *et al*., 2006). Expression of the *Or* gene in white potatoes under the control of a tuber-specific promoter has increased total carotenoid levels to 24 µg/g DW in transgenic tubers and the

sequestering organelles similar to those observed in cauliflower with the *Or* gene mutation were observed. The *Or* gene plays a pivotal role in carotenoid accumulation and stability during post-harvest cold storage of tubers and could complement other metabolic engineering strategies in food crops (Lu *et al*., 2006; Lopez *et al*., 2008; Li *et al*., 2012). Carotenoid levels are associated with major genes or QTLs in various crops like sweet potatoes, melons, citrus and canola, and it has the potential to enhance carotenoid levels when exploited them in staple foods (Bai *et al*., 2011).

In comparison with the major carotenoid genes governing the biosynthetic pathway, the carotenoid cleavage dioxygenase genes are under exploited as their underlying functions are yet to be refined. The dioxygenase (NCED/CCD) family enzymes produce apocarotenoids which have potential applications in plant development, aroma, flavor and human health (Auldridge *et al*., 2006a; Cazzonelli and Pogson, 2010). Functional characterization of NCEDs and CCDs in different plant species has provided an overall spectrum of the enzyme activities involved in apocarotenoid formation from different substrates *via* cleavage at specific positions (Ahrazem *et al*., 2010). Transgenic approaches by RNA interference in white chrysanthemum have functionally validated a gene controlling petal coloration (Ohmiya *et al*., 2009).
The suppression of CCD gene activity may help the accumulation of carotenoid compounds by preventing carotenoid breakdown into apocarotenoids.

### 1.4. Apocarotenoids

Carotenoids are catabolized by the family of carotenoid cleavage dioxygenases (CCDs), which produce apocarotenoids. CCDs are widely distributed in nature that possess significant metabolic functions in diverse organisms. The CCD gene family is responsible for the synthesis of essential apocarotenoids such as vitamin A, phytohormones (e.g. abscisic acid and strigolactones), chromophores, novel signalling molecules, volatiles (β-ionone, geranylacetone) and

colored spices (bixin and saffron; reviewed in Cuttriss *et al*., 2010; Figure 1). ABA is a plant abiotic stress regulator synthesized from cleavage of the carotenoid substrate nine-*cis*-epoxycarotenoid at 11, 12 (11'12') double bonds by the enzyme nine-*cis*-epoxycarotenoid dioxygenase (NCED; Schwartz *et al*., 1997).

NCED is the first characterized member of a gene family isolated from maize *Vp14* (*Viviparous 14)* mutant, which forms $C_{25}$ apo-aldehydes and xanthoxin, a precursor of ABA that upregulates during water-deficit stress conditions (Schwartz *et al*., 1997; Tan *et al*., 1997; Tan *et al*., 2003; Bray 2002). Nine members of the CCD family in Arabidopsis genome (CCD 1, 4, 7, 8 and NCED 2, 3, 5, 6, 9) have sequence homology with the maize *Vp14* gene family but exhibit different substrate specificity and tissue distribution (Schwartz *et al*., 2001, 2003; Tan *et al*., 2003). Five of the AtNCED proteins with high sequence homology to maize VP14 (AtNCED2, 3, 5, 6, and 9) involve in ABA synthesis (especially AtNCED 3; Iuchi *et al*., 2001). NCEDs have been identified from different plant species including tomato (Burbidge *et al*., 1999), bean (Qin and Zeevaart, 1999), avocado (Chernys and Zeevaart, 2000) and cowpea (Iuchi *et al*., 2000). The remaining four Arabidopsis CCD members (1, 4, 7 and 8) possess 9-*cis*-epoxycarotenoid cleavage activity which is either symmetric or asymmetric (Iuchi *et al*., 2001; Bouvier *et al*., 2005; Ohmiya, 2009). CCDs have been identified in plant species such as chrysanthemum, crocus, petunia, rose, osmanthus, citrus, strawberry, melon, apple, peach, grapes, bitter melon, tomato, pea and rice. Recent studies identified a novel dioxygenase called carotenoid cleavage dioxygenase 2 (CCD2) from *Crocus sativus*, which is distinct and closely related to the CCD1 dioxygenase family (Frusciante *et al*., 2014).

### 1.4.1. Subcellular localization of the CCD family

The CCD family is ancient with family members present in bacteria, plants, and animals (Bouvier *et al*., 2005; Kloer and Schulz, 2006; Auldridge *et al*., 2006a). The NCED protein

VP14 in maize mainly targets epoxy-carotenoid substrates located in the plastids (Qin and Zeevaart, 1999; Tan *et al*., 2001). CCD1 orthologues are the only members localized to the cytosol unlike other CCDs and NCEDs. Other CCDs like AtCCD4, AtCCD7 and AtCCD8 are localized onto plastids (Booker *et al*., 2004; Auldridge *et al*., 2006b). The enzyme CCD2 from *Crocus sativus* has a cytoplasmic localization like CCD1 (Frusciante *et al*., 2014).

### 1.4.2. Genomic organization and gene structure of CCDs

The CCD gene members are distinguished by their variation in gene structures across various species. The *CCDs* contain either no introns or many introns (Ahrazem *et al*., 2010). For example, *CCD1* in Arabidopsis and rice contain 11 or 13 introns, respectively (Auldridge *et al*., 2006a). The *CCD7* and *CCD8* contain multiple introns as in plants like Arabidopsis, rice, pea and petunia (Auldridge *et al*., 2006b; Johnson *et al*., 2006; Drummond *et al*., 2009). The maize *CCD8b* is phylogenetically related to *CCD1* but has a unique gene structure distinct from *CCD8a* (Vallabhaneni *et al*., 2010). CCD4 has been reported to contain no introns or may have one or two intron sequences. The genes *CmCCD4a* (*Chrysanthemum morifolium*) and *MdCCD4a* (*Malus domestica*) contain one intron in their genomic DNA whereas *AtCCD4 (Arabidopsis thaliana)* and *RdCCD4 (Rosa damascena)* contain no introns in their genomic DNA although they exhibit similar substrate specificity. The *OfCCD4* (*Osmanthus fragrans*) contain introns but the encoded protein could cleave neither carotenoid nor apocarotenoid compared with other plant CCD4 genes (Ohmiya *et al*., 2006; Huang *et al*., 2009b; Vallabhaneni *et al*., 2010). Genomic analysis has revealed the presence of different *CCD4* in *Crocus* species (Ahrazem *et al*., 2010). *CsCCD4a* and *CsCCD4b* are considered to be allelic variants due to the triploid nature of *Crocus sativus* (Rubio *et al*., 2008). *CsCCD4a* exists with or without introns whereas *CsCCD4b* has a unique intron.

CCD4 enzymes are encoded by at least two forms of genes with different gene structures and genome positions (Ahrazem *et al*., 2010). Variation in the promoter region of *CsCCD4a* and *CsCCD4b* in comparison with other plant species has highlighted the existence, absence and unique presence of *cis* regulatory elements that may participate in plant abiotic stress response conditions (Ahrazem *et al*., 2010; Rubio *et al*., 2008). Similarly, *CsCCD4c* has been identified as intron-less gene expressed only in stigma tissues and up-regulated by environmental stress conditions thus it seems to be involved in apocarotenoid production during adaptation to stress (Rubio-Moraga *et al*., 2014a).

### 1.4.3. Substrate specificity and apocarotenoid production by CCDs

CCDs possess different substrate preferences. Among the CCDs, CCD1 and CCD4 cleave their substrates symmetrically at 9, 10 and 9'10' double bonds, although CCD1 also cleaves at different positions [5, 6 (5', 6'); 7, 8 (7', 8'); 9, 10 (9', 10')] and have multiple substrates. CCD7 can also cleave multiple substrates but cleaves asymmetrically (Schwartz *et al*., 2001; Booker *et al*., 2004; Schwartz *et al*., 2004; Vogel *et al*., 2008; Huang *et al*., 2009a). AtCCD7 cleaves β-carotene, generating $C_{13}$ volatile β-ionone and $C_{27}$ aldehyde 10'-apo- β-carotenal. AtCCD8a catabolizes the $C_{27}$ aldehyde derived from AtCCD7 activity and produces $C_{18}$ and $C_9$ apocarotenoids, respectively (Schwartz *et al*., 2004; Auldridge *et al*., 2006b). Both AtCCD7 and AtCCD8 are required for the synthesis of strigolactone, which controls lateral shoot growth (Booker *et al*., 2004; Schwartz *et al*., 2004). The CsCCD2 from *Crocus sativus* sequentially cleaves 7,8 (7',8') double bonds and converts zeaxanthin into crocetin dialdehyde through a $C_{30}$ intermediate 3-OH-β-apo-8'-carotenal (Frusciante *et al*., 2014).

CCD1 cleaves multiple carotenoid substrates that includes all carotenoids (ζ-carotene, lycopene, β-carotene, zeaxanthin, δ-carotene and lutein) at different positions and produces a variety of apocarotenoids (Vogel *et al*., 2008) although recent studies in golden rice suggest that

apocarotenoids themselves are major substrates of rice CCD1 (OsCCD1; Ilg *et al*., 2010). CCD1 from arabidopsis (Schwartz *et al*., 2001), saffron (Bouvier *et al*., 2003; Rubio *et al*., 2008), petunia (Simkin *et al*., 2004a), tomato (Simkin *et al*., 2004b), grapes (Mathieu *et al*., 2005), melons (Ibdah *et al*., 2006), citrus (Kato *et al*., 2006), coffee (Simkin *et al*., 2008), maize (Vogel *et al*., 2008), strawberry (Garcia-Limones *et al*., 2008), rose (Huang *et al*., 2009a) and osmanthus (Baldermann *et al*., 2010) cleave all *trans*, 9-*cis*-carotenoids and epoxycarotenoids at 9,10 (9', 10') double bond positions. CCD1 from arabidopsis, crocus, strawberry, grapes and tomato symmetrically cleaves zeaxanthin at 9,10 (9', 10') bonds and releases a $C_{13}$-non-risoprenoid called 3-hydroxy- β-ionone (Schwartz *et al*., 2001; Bouvier *et al*., 2003; Garcia-Limones *et al*., 2008; Mathieu *et al*., 2005; Simkin *et al*., 2004a, b). The non-cyclic lycopene is cleaved at 5, 6 (5', 6') double bonds by CCD1 from maize, arabidopsis and tomato (Vogel *et al*., 2008). OsCCD1 cleaves lycopene at 7, 8 (7', 8') position to generate three different volatile compounds such as pseudoionone, 6-methyl-5-hepten-one and geranial (Ilg *et al*., 2009).

CCD4 does not cleave linear carotenoids (lycopene and cis-ξ-carotene) or hydroxyl groups (zeaxanthin and lutein) but predominantly cleaves cyclic non-polar carotenoids (β-carotene) and apocarotenoids. CCD4a from chrysanthemum (CmCCD4a) and apple (MdCCD4a) produces volatile β-ionone upon β-carotene cleavage at 9,10 (9'10') double bonds whereas AtCCD4 from arabidopsis and RdCCD4 from rose preferentially cleaves the $C_{30}$ apocarotenoid, 8'–apo-β-caroten-8'–al (Ohmiya *et al*., 2006; 2009; Huang *et al*., 2009b). Recombinant expression of *CCD4* in *E.coli from Crocus sativus* (*CsCCD4 a, b*), *Rosa damascena* (*RdCCD4*) and *Osmanthus fragrans* (*OfCCD4*), *Malus domestica* (*MdCCD4*) and *Chrysanthemum morifolium* (*CmCCD4*) also proved its substrate specificity and cleavage positions. An additional *CCD4* allele (*CsCCD4c*) from *Crocus sativus* cleaves β-carotene at 9, 10 (9', 10') positions and releases β-ionone and β-cyclocitral at lower levels upon cleavage at 7, 8 (7', 8') positions (Rubio-Moraga *et al*., 2014a). In contrast, zeaxanthin cleavage dioxygenase

(CsZCD) from saffron cleaves zeaxanthin symmetrically at 7, 8 (7', 8') double bonds. Although *CsZCD* is a truncated version of *CsCCD4*, no cleavage of zeaxanthin was obtained on recombinant expression of *CsCCD4* in *E.coli* (Bouvier *et al*., 2003; Rubio *et al*., 2008). Similarly, grape CCD4 (*VvCCD4a,b*) catalyses an additional cleavage at 5,6 (5',6') double bond positions that produces 6-methyl-5-hepten-2-one from substrate lycopene. This suggests the ability of CCD4 to cleave both cyclic and linear carotenoids (Lashbrooke *et al*., 2013).

### 1.4.4. Regulation of Carotenoid turnover by *CCD* genes

The biosynthesis of apocarotenoids by the cleavage activity of CCDs is an important regulatory control over carotenoid accumulation in plants. The NCEDs are involved in ABA synthesis and thus controls abiotic stress (Schwartz *et al*., 1997; Tan, 1997; Iuchi *et al*., 2001; Seo and Koshiba, 2002). The Arabidopsis CCD7 and CCD8 proteins encoded by genes *MAX3* and *MAX4* synthesize carotenoid-derived shoot branching regulators, strigolactones (Booker *et al*., 2004; Bouvier *et al*., 2005; Auldridge *et al*., 2006b). Arabidopsis and pea *CCD8a* are involved in the production of strigolactone, fungal symbiosis and parasitic weed interactions (Gomez-Roldan *et al*., 2008). Co-expression of *AtCCD7* and *AtCCD8* in *E.coli* sequentially cleave β-carotene to produce a $C_{18}$ compound, 13-apo-carotenone (Schwartz *et al*., 2004). Similar high expression of *CsCCD8* combined with *CsCCD7* in saffron axillary buds suppresses its outgrowth, suggesting apical dominance and positive regulation of procambial activity by strigolactones (Rubio-Moraga *et al*., 2014b). Unlike the other CCDs, NCEDs and CCD7/8 do not seem to intensely affect carotenoid levels in plants.

CCD1 and CCD4 are widely responsible for apocarotenoid flavor and aroma volatiles from various plant organs, where loss of function leads to higher carotenoid levels (Auldridge et al., 2006b). The cytosol localized CCD2 cleaves carotenoids in the outer envelope of plastids and catalyses the first dedicated step in the biosynthesis of crocin from *Crocus sativus* (Frusciante *et al*., 2014).

Recombinant expression of CCD1 in *E.coli* cleaves β-carotene into volatile β-ionone in Arabidopsis seeds (*AtCCD1*), tomato fruits (*LeCCD1a,b*); stigma tissues of saffron (*CsCCD1a, b*) and melon fruits (*CmCCD1*; Schwartz *et al*., 2001; Simkin *et al*., 2004a,b; Bouvier *et al*., 2003; Ibdah *et al*., 2006). The plastogobule targeted enzyme CCD4 mediates the formation of fragrant and volatile C13 ketones (β-ionone) cleaved by cyclic carotenoids (Ahrazem *et al*., 2010). Other volatiles such as geranyl acetone, geranial, β-cyclocitral, theaspirone, α-damascenone and β-damascenone also contribute to flavor and aroma of flowers, whose structures reveal the isoprenoid based origin from which the apocarotenoids are produced (Auldridge *et al*., 2006a).

Regulation of CCDs in association with carotenoid levels is primarily at the transcript levels. The genes *CCD1* and *CCD4* have been identified from many crops especially in flowers and fruits, which demostrates their critical role in carotenoid accumulation. *CCD1* or *CCD4* transcript levels in white petals of chrysanthemum, potato tubers, citrus peel, white-fleshed peach, arabidopsis seeds, rose flowers and saffron stigmas proves a negative association with carotenoid content (Ohmiya *et al*., 2006; 2009; Campbell *et al*., 2010; Rodrigo *et al*., 2013; Brandi *et al*., 2011; Huang *et al*., 2009b; Rubio-Moraga *et al*., 2009; 2014a; Frusciante *et al*., 2014). An increase in *FaCCD1* transcript levels in strawberry led to a dramatic decrease in lutein levels during the course of ripening (García-Limones *et al*., 2008). *CCD4* in Arabidopsis is a major determinant in carotenoid turnover, suggesting its role of β-carotene degradation in drying seeds and senescing leaves (Gonzalez-Jorge *et al*., 2013). The white ray petals of chrysanthemum are devoid of carotenoids although all carotenoid genes are generally expressed in its petals (Kishimoto and Ohmiya, 2006). In contrast, the yellow chrysanthemums are rich in carotenoids especially lutein derivatives (Kishimoto 2004; 2007). Chrysanthemum *CCD4* (*CmCCD4a*) was later identified to be responsible for white petal coloration due to its high transcript levels (Ohmiya *et al*., 2006). Suppression of *CmCCD4a* expression by RNA interference (RNAi) in white chrysanthemum transformed the petal coloration to yellow in the

transgenic lines (Ohmiya *et al*., 2009), hence *CmCCD4a* is the key controlling factor in ray petals (Yoshioka *et al*., 2012). Similar down-regulation of *CCD4* in white fleshed potato increased carotenoid content in transgenic potato tubers, elucidating the regulatory role of the gene (Campbell *et al*., 2010). The citrus *CCD4* is responsible for the biosynthesis of $C_{30}$ apocarotenoids which are the key pigments in fruits (Rodrigo *et al*., 2013). The peach *CCD4* has been proved to control white flesh color with abundantly high transcript levels and degrades β-carotene into non-isoprenoid volatiles (Adami *et al*., 2013; Ma *et al*., 2014). Expression of *CCD1* and *CCD4* are not correlated with carotenoid accumulation in flowering plants like morning glory (Yamamizo *et al*., 2010). This explains the fact that CCDs may have a different biological function or other factors may regulate carotenoid levels in some plant species.

### 1.5. Hypothesis

Because the gene *CmCCD4a* is responsible for the white coloration in chrysanthemum petals and release of volatile apocarotenoids on oxidative cleavage (Ohmiya *et al*., 2006; 2009), I hypothesize a similar influence of *CCD4* in Brassica (fast plant) flower petals.

### 1.6. Thesis objectives

Previous studies in chrysanthemum, potato and peach (Ohmiya *et al*., 2006; Campbell *et al*., 2010; Ma *et al*., 2014) have proved that *CCD4* is a key gene that regulates carotenoid levels in various plant organs, which may be of significance in metabolic engineering of carotenoids or apocarotenoids in food crops.

Hence, the objectives of the study fulfilled in Chapter 3 are:

1. Testing the correlation between molecular polymorphism of *CCD4* and phenotypic variation in flower color of Brassica fast plants.
2. Functional characterization of Brassica (fast plant) *CCD4* utilizing biochemical (HPLC, GC-MS) and transgenic (RNA*i*) methods.

**CHAPTER 2**

# MATERIALS AND METHODS

## 2.1 Plant materials and growth conditions

Fast plant seeds of white and yellow flower phenotypes were raised in a greenhouse (USDA-ARS, Cornell University) for crossing and to conduct experiments such as gene expression using qRT-PCR, DNA sequencing, and biochemical analyses using HPLC and GC-MS.

### 2.1.1 Generation of $F_2$ fast plant population

A cross between white and yellow fast plants produced an $F_1$ generation of plants with heterozygous nature. The $F_1$ plants were further selfed to develop an $F_2$ population. Seventy-five F2 plants were grown in medium-sized plastic pots in greenhouse No.5 at USDA-ARS, Robert Holley Centre for Soil, Plant and Nutrition for Human Health, Ithaca, NY. The $F_2$ plants were well maintained through periodic watering, timely application of fertilizers, and removal of dead leaves and periodic spray of pesticides against aphid infestations. Soluble fertilizers were thoroughly mixed with water and applied once a week during the vegetative phase followed by every two weeks from flowering until seed harvest. Slow release nutrient pellets were also applied to soil at the time of sowing to maintain good plant health until the end of the study.

## 2.2 Quantitative real time PCR

### 2.2.1 RNA extraction

Total RNA was extracted from flower samples (50-100mg) of white and yellow fast plant phenotypes using Trizol reagent according to the manufacturer's instruction (Invitrogen, CA). Each of the flower samples was collected and extracted in triplicates. Leaf tissues 50-100 mg were added with 1ml of Trizol reagent to individual samples and homogenized with glass

Teflon or power homogenizer. The contents were centrifuged at 12000 x g for 10 min and the supernatant was transferred to new 1.5 ml tube. The RNA samples were incubated for 5 min room temperature (RT) and added 0.2 ml of chloroform per 1 ml of Trizol reagent used for homogenization. The tubes were vigorously shaken for 15 sec and incubated at 2-3 min RT. The samples were centrifuged at 12000 x g for 15 min at 4°C. The aqueous phase of the sample was removed, transferred to a new tube, added with 0.5 ml of 100% isopropanol, incubated for 10 min at RT and centrifuged at 12000 x g for 10 min at 4°C. The RNA was found visible after centrifugation by associating with gel-like pellet on the side and bottom of the tube. The supernatant from the tube was removed and the pellet that contained RNA was retained. The pellet was washed with 1 ml of 75% ethanol per 1ml of Trizol reagent used for initial homogenization. The samples were then mixed in a vortex briefly and then centrifuged at 7500 x g for 5 min at 4°C. The pellet was air-dried for 5-10 min, re-suspended in 80 µl sterile double-distilled water and stored at -80°C for long term use.

### 2.2.2 RNA quantification

RNA was quantified by NanoDrop spectrophotometer (NanoDrop Technologies). The expected A260/A280 ratio for RNA is generally ~2.0. The RNA quality was checked in 1.5% agarose gel by loading 1 µl of crude RNA and 2 µl of 6X loading dye (Bromophenol Blue and Xylene Cyanol FF).

### 2.2.3 cDNA synthesis

cDNA synthesis was carried out using RNA of flower tissues. Reverse transcription of total RNA (500ng-1 µg) was carried out using oligo-dT (Promega, Madison, WI) primer and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) to generate the first strand cDNA template. cDNA synthesis was done by adding 1 µl oligo$(dT)_{18}$ (500ng/µl), 10 pg-5 µg total RNA/10 pg-500 ng mRNA, 1 µl dNTP (10 mM ) and made up to 20 µl with sterile

distilled water. The mixture was heated to 65°C for 5 min and immediately incubated in ice for at least a minute. Then, the proportional amount of master mix containing 5X First-Strand Buffer (4 µl), 0.1 M DTT (1 µl), RNase OUT Recombinant RNase Inhibitor (0.2 µl) and SuperScript III RT (0.2 µl) was added to the initial product. The final contents were thoroughly mixed and incubated at 50°C for 1 hour. The reaction was ended by 70°C incubation for 15 min. The cDNAs were further diluted 10-fold for use in qRT-PCR reactions. Synthesized cDNA samples were stored at -20°C.

### 2.2.4 Sample preparation and Thermo-cycling conditions

Diluted cDNA (1µl) from flower samples of white and yellow fast plants were used as templates in qRT-PCR reaction, which contained SYBR Green PCR master mix and run on Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The primer sequences for the fast plant genes *CCD4a* and *CCD4b* (Table 1) were designed using the available *Brassica oleracea* (cauliflower) sequence data from NCBI database. Thermo-cycling program was set up, starting with initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing and extension at 60°C for 1 min.

### 2.2.5 Normalization of gene expression by ΔΔCt method

The relative expression level was calculated by ΔΔCt method as described by Lyi *et al*., 2007. Each of the cDNA samples prepared with 2-3 biological repeats were run in duplicates. *Actin* gene from cauliflower (*BoActin*) was used as an endogenous control. The relative gene expression was quantified from $C_t$ values obtained for samples and endogenous control. The average Ct values were obtained by calculating the mean of both treatment and control sample duplicates. Further, treatment and control Ct values were obtained. Control $C_t$ was calculated by the difference between the average Ct values of white *CCD4a* and white *BoActin*, *i.e*., W (ccd4a) - W (actin). Similarly, the difference between white *CCD4b* and white *BoActin* was calculated.

**Table 1: List of quantitative RT-PCR primers**

| Gene | Primer Orientation | Primer sequence |
|---|---|---|
| *CCD4a* | Forward | 5'- CTGCAGCAGATACGTCAAGACT-3' |
| | Reverse | 5'- GTTCGCTAAACCAATGCCGTTGAT-3' |
| *CCD4b* | Forward | 5'- TATAATGGATCTGGTGCTCGAGGG-3' |
| | Reverse | 5'- TTCCATCGTTTTCATCCCAAGCA-3' |
| *BoActin* | Forward | 5'- CCGAGAGAGGTTACATGTTCACCAC-3' |
| | Reverse | 5'- GCTGTGATCTCTTTGCTCATACGGTC-3' |

Treatment Ct was then calculated by the difference between the average Ct values of yellow *CCD4a a*nd yellow *BoActin*. The difference between yellow *CCD4b* and yellow *BoActin* was also obtained. Both control ΔCt and treatment ΔCt were calculated for each sample replicate and hence ΔΔCt value was calculated by the difference between the average treatment ΔCt and the average control ΔCt. The Relative gene expression ratio was finally derived using the equation, $R = (2)^{-\Delta\Delta Ct}$ (Pfaffl, 2001) and the formula used in calculation, $R= (\Delta Ct)^2$. The fast plant *CCD4* gene expression was thus normalized to endogenous *Actin*. The data was represented in bar graph inclusive of standard error (SE) by customizing the standard deviation (SD) calculated for the treatment $C_t$ and control $C_t$ respectively.

### 2.3 Molecular analysis of the candidate gene *CCD4*

#### 2.3.1 Isolation of genomic DNA

DNA extraction of fast plant phenotypes (white and yellow) were extracted by the method used by Lu *et al*., 2006.

##### 2.3.1.1 Extraction of genomic DNA

Fresh young leaves with an area of 1- 2$cm^2$ was cut and placed in a microcentrifuge tube. The tubes frozen in liquid nitrogen were crushed in a mechanical tissue lyser and added with 750 µl extraction buffer to each sample, well mixed and placed at 65°C for 10 min. The samples were added with 150 µl 5M KOAc, mixed well, ice incubated for 15 min and centrifuged at 12000 rpm for 10 min. The supernatant obtained was transferred to a new 1.5 ml microcentrifuge tube, added with 850 µl propan-2-ol, mixed well, stored at 4°C overnight and centrifuged at 12000 rpm for 5 min. The supernatant was poured off and the pellet was washed with 950 µl of 70% ethanol followed by centrifugation at 12000 rpm for 5 min. Finally, the pellet was air-dried and re-suspended with 80-100 µl of TE buffer containing 2 µl of RNaseA in order to avoid RNA contamination.

### 2.3.2 PCR amplification of gene *CCD4*

PCR amplification was carried out using white (W) and yellow (Y) fast plant DNA and over-expression primers designed from cauliflower *CCD4a* and *CCD4b* sequences. The cauliflower *CCD4* sequence (CDS) length approximates to 1.8 kb. Highly conserved over-expression primer pair, Ox-2F (5'-ACTCTCTTTCTTCCTCTTCCTTC-3') and Ox-2R (5'-GGTCACTCTCCTTCACAAATAG-3') was designed using Oligoanalyzer 3.1 (http://eu.idtdna.com) considering necessary parameters suitable for specific binding to the DNA template. The PCR cocktail per 20 µl reaction volume was prepared using sterile double-distilled water, 2.5mM dNTP (2.0 µl), 10X ExTaq buffer (2.0 µl ), ExTaqR (5 units/µl; TAKARA BIO Inc., USA), forward and reverse primers each of 1.0 µl with 10 µM concentrations. The cocktail was finally added to template DNA (50-70 ng). The PCR program run in BIORAD's PTC-100 was set up with initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 30 sec, extension at 72°C for 2 min. Final extension was ended at 72°C for 10 min and preferential hold at 4°C.

### 2.3.3 Cloning and DNA sequencing

The PCR products were checked on 1.5% agarose gel and DNA bands were cut under a UV trans-illuminator. The gel was purified using Qiaquick gel extraction kit. The purified PCR product was ligated into pCR®2.1 cloning vector (Invitrogen). A 5 µl ligation reaction volume was set up with overnight incubation at 16°C. The amount of PCR product needed to ligate into 50 ng of pCR®2.1vector at 3:1 molar ratio was calculated based on insert DNA concentration. The concentration of DNA was checked in Nanodrop Spectrophotometer (Nanodrop technologies). Ligation cocktail per 5 µl reaction included insert DNA (2-3 µl), vector (1.0 µl), 10X ligation buffer (0.5 µl) and T4 DNA ligase (0.5 µl). The ligated product (5 µl) was then transformed into commercially available TOP10 *E. coli* competent cells.

The *E.coli* strain TOP10 (Invitrogen Corporation, San Diego, CA) was grown in Luria-Bertani (LB) medium (Sambrook *et al*., 1989) at 37°C in darkness on a platform shaker at 225 cycles per min (Cunningham *et al*., 1996) and stored at -80°C for long term use. The ligated product was transformed by heat shock transformation method described by TA cloning kit instruction manual (Invitrogen). Competent cells (50-100 µl) were added to 5 µl ligated product and incubated on ice for 15-20 min. Then, the cells were exposed to heat shock at 42°C water bath for 30 sec followed by immediate incubation in ice for 2 min. After incubation, 150-250 µl liquid LB was added to the transformed cells and incubated at 37°C, 250 rpm for 1 hour. Meanwhile, LB/kanamycin (50 µg/ml) plates were spread with 40 µl X-Gal (40 mg/ml) for blue-white selection of colonies. The transformed cells were spread on LB /kanamycin 50µg/ml/ X-Gal plate using well sterilized L-rod and incubated at 37°C overnight. Positive white colonies were selected for colony PCR, plasmid isolation, restriction digestion and sequencing.

Fifty-four selected white colonies of both white and yellow fast plant clones were subjected to colony PCR using M13-F and M13-R primers (1.0 µl each), Takara LA Taq (5 units/ µl; Clontech), 10X LA Taq buffer (2.0 µl), dNTP ( 2.0 µl), DNA (single colony) and dd$H_2O$ made up to 20 µl volume per reaction. PCR program was set up starting with initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 30 sec and extension at 72°C for 2 min. Final extension ended at 72°C for 10 min. The colony PCR positive colonies were grown overnight at 37°C in 4ml LB broth +4 µl kanamycin (50 µg/ml) culture medium for plasmid DNA extraction using Qiagen's Miniprep kit. The isolated plasmid DNA was utilized for EcoRI restriction digestion which included plasmid DNA (4.0 µl), 10X NEB EcoRI Buffer (2.0 µl), EcoRI enzyme (0.5 µl) and ddH2O made up to 20.0 µl volume per reaction. The contents were incubated at 37°C for 1 hour and the digested products were loaded on to 1.2% agarose gel. Positive clones were sequenced for molecular analysis of genes *CCD4a* and *CCD4b* from both the flower color types.

The clones were sequenced at Life Sciences Core Laboratories Center (CLC), Cornell University. The retrieved sequence data was analysed using DNASTAR Lasergene 7.0 software package, which includes various essential tools such as SeqmanPro, Megalign, EditSeq and so on. SeqmanPro was used to assemble the contiqs of the selected clones sequenced using universal primers. The contiqs were then categorized into different groups based on sequence identity in particular set of clones. Then, the group data was aligned using Megalign tool by ClustalW method. The alignment report, sequence distances (% identity and % divergence) and phylogenetic tree were constructed by Megalign tool to highlight the sequence polymorphism between the groups. The phylogenetic tree was also developed with representative members of *CCD4* obtained from GenBank (http://www.ncbi.nlm.nih.gov/). All the four *CCD4* sequences were checked for Open reading frame (ORF) reads and translated into amino acid sequences using Editseq tool.

### 2.3.4 Genome walking

Genome walking procedure was carried out using Clontech Genome walker Universal kit to obtain promoter sequences for *CCD4a* and *CCD4b* fast plant flower color phenotypes. The aim was to identify new SNPs in the promoter to develop markers for co-segregation analysis with the phenotype. According to the kit protocol, genome walker adaptors (reverse primers) were designed from fast plant *CCD4b* (white) sequence, which was expected to target the upstream promoter region with an amplicon size of 2.0 kb. Hence, the walker adpators GTTACTGATCGGAGATCGTTCTTCGGA and GTTAGTCCGAGTGTAGAGTTTCTTATA were used to amplify the isolated DNA from white phenotype for TAIL-PCR reactions and the obtained product was cloned for sequencing.

### 2.3.5 PCR amplification of fast plant *CCD4b* promoter

An alternative method to genome walking was performed to identify promoter sequence from already published scaffold numbers of white *CCD4a* (Bol009345:Scaffold000234:140030:141808) and *CCD4b* (Bol029878:Scaffold000063:815255:824278) from the Brassica database (brassicadb.org/). The coding sequences were obtained in the given scaffold region of *B.oleracea* (Chromosome v1.0) genome using a Genome Browser tool and aligned them with our *CCD4*a and *CCD4b* sequences of white phenotype. Sequence identity was checked using Megalign tool (DNASTAR, Lasergene 7.0). A 2.0 kb promoter sequence was also obtained from the upstream of the *CCD4a* and *CCD4b* scaffold region. Forward and reverse primers (Table 2) were designed only from *CCD4b* promoter and fast plant yellow *CCD4b* coding sequence obtained. PCR conditions were set up as in Method 2.3.2. The expected amplicon sizes using the primer pairs (Table 2) in different combinations were 1.0 and 2.0 kb, respectively.

## 2.4 Functional Characterization of gene *CCD4*

### 2.4.1 Design of RNAi construct for the gene *CCD4b*

RNAi (knock-out) construct was designed using the vector pCAMBIA1300S (8958 bp) for the introduction of 1.1 kb *CCD4b* fragment. Sense and anti-sense primers (Table 3) were designed from yellow flower phenotype of fast plant *CCD4b* sequence and cloned into the binary vector (pCAMBIA1300S). The *CCD4b* fragments were cloned using specific restriction enzymes (Table 3) available in the polylinker region and absent in the insert DNA sequence. The absent sites in the whole gene sequence were identified using Seqbuilder tool (DNASTAR Lasergene 7.0 package). PCR amplification of gene fragments per 50 µl reaction volume was prepared using sterile double-distilled water, 2.5mM dNTP (4.0 µl), 10X ExTaq buffer (5.0 µl ), ExTaqR (0.5 µl; TAKARA BIO Inc., USA), forward and reverse primers each of 2.0 µl with

**Table 2: List of PCR primers used for *CCD4b* promotor amplification**

| Primer | Orientation | Primer sequence |
|---|---|---|
| Promoter 4b | Forward | 5'- ACCTACACGATGAAGCCTGTGAGG -3' |
| | Reverse | 5'- AAACGATGTAGATGGAGGAGGAG -3' |
| Promoter 4b (1) | Forward | 5'- CTCGAAGTAAGGGTTCAAAAACG - 3' |
| | Reverse | 5'- GCCGTTACTAGTGATCCAGCGACC -3' |
| CCD4bY cds | Reverse | 5'- GGTCGCTGGATCACTAGTAACGGC -3' |

The anti-sense PCR fragment (390 bp) along with the internal spacer (230 bp) was cloned initially into a TA cloning vector (pCR®2.1) and transformed into TOP10 *E. coli* competent cells (Invitrogen). Positive colonies were screened through colony PCR using the anti-sense

10 µM concentrations. The cocktail was finally added to template DNA (50-70 ng). The program was run in BIORAD's PTC-100 was set up with initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 2 min, annealing at 60°C for 30 sec, extension at 72°C for 2.30 min. Final Extension was ended at 72°C for 10 min and preferential hold at 4°C. Primers described in Table 3. The presence of insert DNA was further confirmed through restriction enzyme digestion with EcoRI and XhoI. The extracted plasmid DNA of positive clones (pTA-620) was excised from TA vector and introduced into pCAMBIA 1300S containing constitutive promoter CaMV 35S (Zhou *et al*., 2009). The process of restriction digestion in empty vector pCAMBIA 1300S and pTA-620 was digested with KpnI and SacI, respectively. The 10X enzyme buffer solutions numbered from 1 to 4 were used for specific restriction enzymes listed by NEB biolabs (http://www.neb.com/). A 40 µl reaction volume with vector pCAMBIA1300S (6 µl), 10X NEB buffer 1 (4 µl), BSA (0.4 µl), KpnI (1.0 µl), SacI (1.0 µl), dd$H_2O$ (28 µl) and pTA-620 (6 µl), 10X NEB buffer 1 (4 µl), BSA (0.4 µl), KpnI (1.0 µl), SacI (1.0 µl) and dd$H_2O$ (28 µl) was mixed well in individual microcentrifuge tubes and incubated at 37°C for 1.5-2 hours. The digested insert from pTA-620 was again extracted, purified and ligated into the digested empty vector purified by ethanol precipitation/ clean-up method. The ligation reaction volume of 10 µl was added with vector (pCAMBIA1300S; 4.0 µl), Insert (620 bp; 5.0 µl), 10X ligation buffer (0.5 µl) and $T_4$ DNA ligase (0.5 µl) and incubated at 16°C overnight. The ligated product was then transformed into *E. coli* competent cells and incubated overnight at 37°C. White colonies of transformed cells (pCAMBIA1300S-620) were screened through colony PCR using M13-F and M13-R primers.

Positive colonies were selected for plasmid DNA isolation and the presence of insert (620 bp) in pCAMBIA1300S was confirmed through restriction digestion with KpnI and SacI.

To the contrast, the sense fragment (390 bp) was directly cloned into vector pCAMBIA1300S-620 by restriction digestion of p1300S-620 (vector) with BamHI and PstI. A 40 µl reaction volume with vector (6 µl), 10X NEB buffer 3 (3 µl ), BSA (0.3 µl ), BamHI (1.0 µl), PstI (1.0 µl) and dd$H_2O$ (18.7 µl) was set up with incubation at 37°C for 1.5 hours. The digested vector was purified by ethanol precipitation (clean-up) method. The insert DNA with product size 390 bp was PCR amplified (PCR program similar to antisense fragment amplification), gel extracted, purified, ligated into pCAMBIA1300S-620 and incubated overnight at 16°C. The ligated product was then transformed into *E.coli* competent cells ,spread onto LB Agar/kanamycin (50µg/ml) and incubated at 37°C overnight. Fifty-six colonies were screened for positives through colony PCR using the sense forward primer (Table 3) and CaMv35S terminator. Hence, both sense and anti-sense fragments introduced into pCAMBIA1300S were confirmed by restriction digestion with SacI and PstI followed by *Agrobacterium*-mediated transformation.

The *Agrobacterium* (strain GV3101) competent cells were stored at -80°C and thawed on ice before electroporation. The plasmid DNA isolated from pCAMBIA1300S-1.1 kb (1 µl) was added to 100 µl of Agrobacterium competent cells and mixed well. These contents were immediately transferred to pre-chilled electroporation cuvette with 2 mm gap sized. The cuvette holder was also pre-chilled and the gene pulser unit was adjusted by setting the voltage to 2.5 kV, capacitance to 25 µFD and resistance set to 400Ω. The Agrobacterium suspension was electroporated in 9 milliseconds followed by addition of 1 ml of liquid LB and immediate hold on ice. Then, the 200 µl suspension was spread onto LB Agar/Kanamycin (50 µg/ml) /Rifampicin (100 µg/ml) and incubated at 30°C for 48 hours for optimal growth of Agrobacterium colonies harboring pCAMBIA1300S-1.1kb (Figure 2). Four colonies were selected, isolated plasmid DNA,

back transformed into *E.coli* and digested with sense and anti-sense reverse primers (Table 3) to confirm the presence of insert DNA integrated into Agrobacterium. Further, glycerol stocks were prepared for selected colonies that contained *CCD4b* RNAi construct (Figure 2). The sequence data was checked for sequence similarity using blastn (NCBI) and were aligned with yellow *CCD4b* fast plant sequence using Megalign (DNASTAR Lasergene 7.0).

**Table 3: List of primers used for cloning knock-out (RNAi) construct in pCAMBIA1300S**

| S.No. | Primer Name | Restriction site | Primer sequence | Product size |
|---|---|---|---|---|
| 1 | Anti-sense Forward | KpnI | 5'-GGTT**GGTACC**TGGCTAATACGAGTCTAGCT-3' | 620 bp |
| 2 | Anti-sense Reverse | SacI | 5'-CTTC**GAGCTC**TCCACCAATGAGTGAACAAG-3' | |
| 3 | Sense Forward | BamHI | 5'-GTTT**GGATCC**TTCCGGTTTGATTCAACCGG-3' | 390 bp |
| 4 | Sense Reverse | PstI | 5'-TTTT**CTGCAG**TCCACCAATGAGTGAACAAG-3' | |

*The bold underlined letters indicate the recognition sequence of the unique restriction enzymes

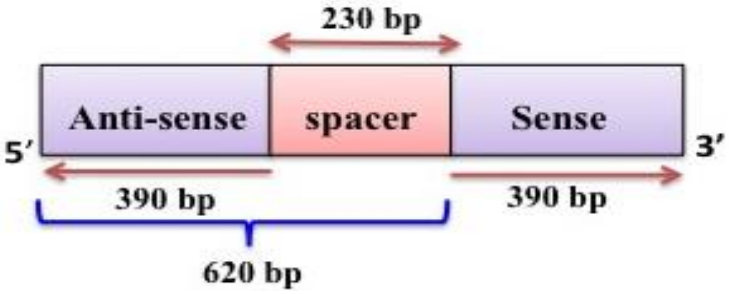



**Figure 2: Design of fast plant *CCD4b* yellow *RNAi* sequence (1.1 kb) amplified using Table 3 primers**

### 2.4.2 Functional complementation of the candidate gene *CCD4* in *E.coli*

The four *CCD4* gene sequences from fast plant flower types *CCD4a* TO1000 (white), *CCD4a* Early big (yellow),*CCD4b* TO100 (white) and *CCD4b* Early big (yellow) were cloned into pETBlue-1 vector (3.476 kb) by Dr. Maria Laura, Agriaquaculture Nutritional Genomic Centre (CGNA). The vector named pAC-Beta (10.6 kb) designed by Cunningham *et al*., 1996 (kindly provided by Dr. F.X.Cunningham, University of Maryland, College Park, MD) was transformed into TOP10 *E.coli* competent cells (Invitrogen Corporation, San Diego, CA). The *CCD4a* and *CCD4b* clones from fast plant white and yellow phenotypes (*BoCCD4aW*, *BoCCD4aY*, *BoCCD4bW* and *BoCCD4bY*) generated in my study were also transformed into β-carotene producing *E.coli* competent cells along with pETBlue-1 constructs for biochemical analyses.

#### 2.4.2.1 pAC-Beta competent cell preparation

The preparation of competent cells was carried out as described in the web link (http://2010.igem.org/ wiki/images/b/bc/IGEM_2010_protocol.pdf). The vector pAC-Beta was transformed into *E.coli* competent cells and spread on LB Agar with chloramphenicol (50 µg/ml). A single colony isolated from the plate was again inoculated in 25 ml LB broth with chloramphenicol (50 µg/ml). The culture was grown at 37°C darkness overnight in a shaker. Then, 1 ml overnight culture was inoculated in 100 ml of LB broth contained in a 250 ml conical flask. The 100 ml culture was kept in a shaker at 37°C for 4 hours until the OD value at 600 nm reached 0.4.The culture was immediately placed on ice for 10 min. The various materials such as solutions, centrifuge, autoclaved glasswares, pipette tips, falcon tubes and micro-centrifuge tubes were pre-chilled. The culture was therefore transferred into two pre-chilled 50ml falcon tubes centrifuged at 2700x g for 10 min at 4°C. The supernatant was removed and the cell pellet was re-suspended in 1.6 ml ice cold 100 mM $MgCl_2$-$CaCl_2$

solution by swirling on ice gently. The cell contents were incubated on ice for 30 min and centrifuged at 2700x g for 10 min at 4°C. The pellet was mixed with 1.6 ml ice-cold 100 mM $CaCl_2$ solution and incubated on ice for 20 min. The cells were combined to one 50 ml falcon tube, added 0.5 ml ice-cold 80% glycerol and mixed well. Aliquots of 100 µl were frozen in liquid nitrogen and stored at -80°C. The *CCD4* gene constructs were finally introduced into β-carotene accumulating *E.coli* competent cells by heat shock transformation method.

#### 2.4.2.2 Extraction of carotenoid pigments from *E.coli* pAC-Beta constructs

Carotenoids were extracted from *E.coli* cells as described by Cunningham *et al*., 1996 with slight modifications. A 0.5-ml aliquot of overnight culture was inoculated in 60 ml of LB medium in a 250 ml Erlenmeyer flask. The *E.coli* cultures with pAC-Beta *CCD4* inserts along with empty vector pAC-Beta as control were grown for 48 hrs in darkness at 25°C, 225 cycles/ min and then harvested by low-speed centrifugation in 50 ml disposable conical centrifuge tubes. The cell density was measured at 600nm under Beckman's UV-Vis Spectrophotometer taking 1ml in a quartz cuvette. The pellets were re-suspended with 0.5 ml water and added with 4.5 ml of 6% KOH in methanol. The tubes were tightly capped and stored in darkness at 37% for 2 hrs. After saponification, insoluble materials were pelleted by centrifugation.
The pellets were extracted with methanol-diethyl ether (1:1 [v/v]) followed by extra addition of diethyl ether until the pellets turned colorless. Further, the extracts were pooled, added with few millilitres of aqueous 5 M NaCl and transferred to diethyl ether. Finally, 1ml diethyl ether extracts were evaporated to dryness under a gentle stream of nitrogen gas.

#### 2.4.2.3 HPLC analysis of carotenoid pigments

The carotenoid pigments extracted were dissolved in 200 µl of ethyl acetate and 40 µl of it were analysed on a Spherisob ODS2 (5 µm particle size) reversed-phase C18 column using a Waters HPLC system equipped with a photodiode array detector (Waters, Milford, MA).

The pigments were separated by a linear gradient between solvent A (90% solution of acetonitrile: $H_2O$: triethylamine at 9:1:0.01, 10% ethyl acetate) and solvent B (80% ethyl acetate, 20% solution of acetonitrile: $H_2O$: triethylamine at 9:1:0.01) over 18 min at a flow rate of 1.0 ml/min. The elution program used: 0 min at 100% A, 0% B; 1-9 min with a linear gradient to 0% A and 100% B; 9-14 min at 0% A and 100% B; 14-18 min at 100% A and 0% B. Identification of β-carotene peak in the bacterial samples was achieved based on absorption spectrum at 453 nm and 14 min retention time obtained for the external β-carotene standard (Sigma-Aldrich, St. Louis, MO).

**2.4.2.4 Determination of volatile β-ionone emission from bacterial headspace through GC-MS**

The β-ionone volatile compound produced from four bacterial pAC-Beta *CCD4* constructs and control (pAC-Beta only) in the headspace were subjected to GC-MS analysis. The sample preparation and running conditions were performed as described by Ibdah *et al*., 2006 with slight modifications. Overnight *E. coli* culture (1 ml) was inoculated in a screw capped headspace vial (Supelco, Bellefonte, PA) containing 4 ml LB, chloramphenicol (50 µg/ml), ampicillin (100 µg/ml) and 0.1 mM IPTG. The cell density was measured at 600nm ($OD_{600}$ = 1.0) before inoculation in vials. Each of the sample cultures was prepared in duplicate for analysis. The screw caps with septa incubated at 37°C for 20 hrs shaken at 250 rpm. The sample cultures in vials were kept on ice and re-activated at 37°C water bath before exposing the SPME fiber into the vials. Headspace volatiles were exposed to SPME fiber for 5 min and inserted into GC injection port using 0.75 mm inlet liner (Supelco). Inlet liner was left in the injection port for 20 min to thermally desorb the electrolytes. The volatile compound β-ionone was analysed from the headspace on an Agilent 6890N network GC system coupled with 30mm x 0.25 mm fused-silica capillary column (J&W scientific). Helium was used as carrier gas with 1.0 ml/min. Injector temperature was set to 250°C for splitless

injection. The oven temperature was set to 50°C for 1 min and then the temperature was ramped up to 200°C at the rate of 4°C/min. MS using a Joel JMS-GC Mate II GC-MS system was utilized for the identification of volatile β-ionone. Chromatograms and mass spectra were analysed using GCmate Pro-3.0 program. Every sample was run for 40 min and a blank run for 20 min was given to desorb all the residual volatiles present in the SPME fiber. Identification of volatile β-ionone released from the bacterial headspace was standardized based on retention time (26.92 min) and mass spectra (177 m/z) obtained for the external standard β-ionone (96%; Sigma-Aldrich, St. Louis, MO).

**CHAPTER 3**

# RESULTS AND DISCUSSION

## 3.1. Flower color in Brassica (fast plant) is monogenic

It is evident that flower color in chrysanthemum was monogenic due to the dominance pattern of white color over yellow (Hattori, 1991). Later, the carotenoid cleavage dioxygenase gene (*CmCCD4a*) proved to be responsible for white petal coloration in chrysanthemum (Ohmiya *et al*., 2006). Similarly, white petal coloration in *Ipomoea sp*. was predicted dominant over yellow, however the progenies of the cross showed yellow (Yamamizo *et al*., 2010). Flower color in Brassica (fast plant) evolved phenotypically into white and yellow types (Figure 3). A cross between white (W) and yellow (Y) fast plants developed $F_1$ generation of plants which had white petal-colored phenotype. $F_2$ progenies were generated by selfing $F_1$ plants and seventy-five F2 plants were grown whose phenotypic observations were taken at the time of flowering. The $F_2$ segregation ratio is generally represented as phenotypic (3:1) and genotypic (1:2:1) for monogenic Mendelian traits. Fifty-four $F_2$ plants showed white flower color in contrast to the remaining twenty-one that exhibited yellow. According to $\chi^2$ test, the observed phenotypic ratio was found non-significant and equal to the expected Mendelian ratio (3:1) thus explained the dominance of single gene controlling flower color in fast plants (Table 4). Hence, the result suggested that flower color in Brassica Fast Plant (FP) is monogenic and *CCD4*- like gene may influence petal coloration as that in chrysanthemum.

**Figure 3: Flower colors in Brassica fast plants**

**Table 4: Phenotypic observation of $F_2$ fast plant population developed from a cross between white (W) and yellow (Y) color Brassica fast plants**

| Test lines | Observed no. of plants (O) | Expected no. of plants (E) | $(O-E)^2$ | $\chi^2$ value $(O-E)^2/E$ |
|---|---|---|---|---|
| White | 54 | 56.25 | 5.0625 | 0.09 |
| Yellow | 21 | 18.75 | 5.0625 | 0.27 |
| | | | | **0.36** |

*Based on $\chi^2$ distribution table, the calculated $\chi^2$ value of 0.36 is less than the critical value of 3.84 for probability (p) equal to 0.05, hence there is no significant difference between the observed and expected mendelian ratios.

### 3.2. Expression of *CCD4a* and *CCD4*b gene transcripts in fast plant flower tissues

It has been reported that *CmCCD4a* transcripts showed relatively high expression in all white flowered chrysanthemums but were negligible in yellow cultivars (Ohmiya *et al*., 2006). Thus, the expression pattern of fast plant *CCD4a* and *CCD4b* was examined in white and yellow petal color Brassica fast plants. The RNA of flower samples were quantified and cDNA synthesis was performed for gene expression analysis. The qRT-PCR analysis revealed similar levels of *CCD4a* transcripts in white flower and yellow flower of Brassica fast plants (Table 5; Figure 4). A 250-fold decrease in *CCD4b* transcript levels was observed in yellow phenotype compared with white petal color, hence proved evidence to predict that *CCD4b* may be responsible for petal coloration in fast plants (Table 5; Figure 4).

Ohmiya (2009) questioned the mechanism of white petal coloration in chrysanthemum is similar to other plants or not. Recently, it has been revealed that peach *CCD4 t*ranscript levels correlated with carotenoid content, thus, suggesting that *CCD4* is responsible for white coloration of peach fruit flesh (Ma *et al*., 2014). Other *CCD4* orthologs like *Scutellaria baicalensis* (*SbCCD4*), *Momordica charantia* (*McCCD4*), *Osmanthus fragrans* (*OfCCD4*), *Crocus sativus* (*CsCCD4*), *Rosa damascena* (*RdCCD4*), *Malus x domestica* (*MdCCD4*) and *Arabidopsis thaliana* (*AtCCD4*) have been reported to have high expression in flowers or floral organs (Tuan and Park, 2013; Tuan *et al*., 2013; Han *et al*., 2014, Rubio *et al*., 2008; Huang *et al*., 2009b; Amponna-Dwamena *et al*., 2012; Ahrazem *et al*., 2010). On the other hand, *CCD4* expression in *Ipomoea sp*. proved no role in carotenoid degradation as its transcripts drastically reduced during petal maturation stage in white flowers (Yamamizo *et al*., 2010). Hence, the mechanism involved in petal coloration appears unique in different plant species. According to Huang *et al*., (2009b), this may be due to differential expression of *CCD4* isoforms exhibiting different biochemical functions.

**Table 5. Real time-PCR analysis for *CCD4a* and *CCD4b* gene transcripts from fast plant flower color cDNA**

| | Average $C_t$ | | Average | Relative gene expression $(\Delta Ct)^2$ | |
|---|---|---|---|---|---|
| **Sample** | **Treatment (T)** | **Control (C)** | **T-C** | **CCD4a** | **CCD4b** |
| White | 11.039 | 10.902 | 0.137 | 1 | 1 |
| Yellow | 12.325 | 4.292 | 8.033 | 0.909 | 0.004 |

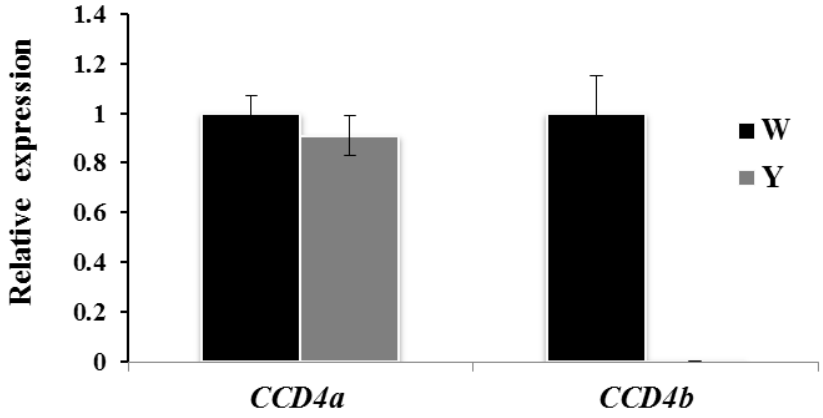



**Figure 4: Expression profiling of alleles *CCD4a* and *CCD4b* in flower petals.** qRT–PCR was performed using white (W) and yellow (Y) fast plant flower cDNA using gene specific primers and *Actin* gene as endogenous control for normalization. Samples were run in duplicate and prepared with two biological repeats. Error bars represent SD.

### 3.3. Isolation and Identification of fast plant *CCD4*

The genes *CCD4a* and *CCD4b* were isolated from fast plant genomic DNA of white and yellow phenotypes using a simple PCR reaction. The amplicons were further cloned and sequenced for gene(s) identification (Figure 5). The sequence data for the selected clones (Table 6a) were analysed to identify nucleotide polymorphisms between the flower color types and also the homology between fast plant *CCD4* genes and other orthologs. The sequences of the selected clones were grouped to form contigs. The contig groups were assembled to four *CCD4* nucleotide sequences *BoCCD4aW*, *BoCCD4aY*, *BoCCD4bW* and *BoCCD4bY* from white and yellow plasmid clones (Table 6b). These four sequences were further aligned using BLOSUM62 matrix with ClustalW (Figure 6a). The fast plant white and yellow *CCD4a* (*BoCCD4aW* & *BoCCD4aY* ) showed 99.8% nucleotide sequence identity. Similarly, *BoCCD4bW* and *BoCCD4bY* exhibited 99.8% identity between the flower color types (Figure 6a, b). The *CCD4* genes from white (*BoCCD4aW* & *BoCCD4bW*) and yellow (*BoCCD4aY* & *BoCCD4bY)* flower colors displayed 88.0% and 87.0% similarity between each other (Figure 6b). Since the sequences of *BoCCD4a* and *BoCCD4b* were found similar within the exon region; variations in the gene promoter regions might substantiate the level of *BoCCD4b* gene expression between white and yellow petal color varieties (Figure 4).

The fast plant nucleotide sequences (*BoCCD4aW*, *BoCCD4aY*, *BoCCD4bW* and *BoCCD4bY)* were also compared for sequence similarity with cauliflower, its close relative (Figure 6b) . The *CCD4a* alleles of the white and yellow petal color varieties (*BoCCD4aW* & *BoCCD4aY*) showed 98.4% and 98.5% similarity with cauliflower *CCD4a* sequence, respectively. Similarly, *BoCCD4bW* & *BoCCD4bY* have 97.2% and 97.1% similarity with cauliflower *CCD4b* sequence, respectively (Figure 6b). The *CCD4* orthologs from different plant species were compared with fast plant *CCD4* genes, *CCD4a* and *CCD4b* based on sequence distance

matrix (% identity and divergence). The *CCD4a* and *CCD4b* from fast plant white and yellow-colored flower phenotypes displayed 84 % similarity with *Arabidopsis thaliana AtCCD4* (Figure 6b). Except *AtCCD4*, all other plant *CCD4*s (Table 7) have approximately 62.5% similarity with fast plant *CCD4a* and *CCD4b* from both the flower colors (Figure 6b). The sequence size (~ 1.8 kb) of fast plant *CCD4a* and *CCD4b* was observed no difference thus appeared intron-less like *Arabidopsis thaliana* and *Rosa damascena CCD4*s (Huang *et al*., 2009b; Ahrazem *et al*., 2010). Other species such as *Chrysanthemum morifolium*, *Lycopersicon esculentum*, *Osmanthus fragrans*, *Malus domestica*, *Crocus sativus* contain introns with different intron lengths and intron numbers within the coding regions. Thus, the gene structure of *CCD4* is dynamic and non-conserved in plants. Although the CCD gene family in plants and animals possess several introns unlike the NCED group, there is an independent loss of introns (>89%) in the genomic sequence of few species, which suggested the presence of an ancestral intron in *CCD4* (Tan *et al*., 2003; Ahrazem *et al*., 2010; Rubio *et al*., 2008). The presence of introns in *CCD4*s of chrysanthemum, apple and osmanthus may have functional importance (Huang *et al*., 2009b). Similarly, the intron-less Arabidopsis *NCED3* proved responsible for ABA synthesis under stress (Tan *et al*., 2003). Hence, lack of introns in fast plant *CCD4* may have a potential function in flower petals.

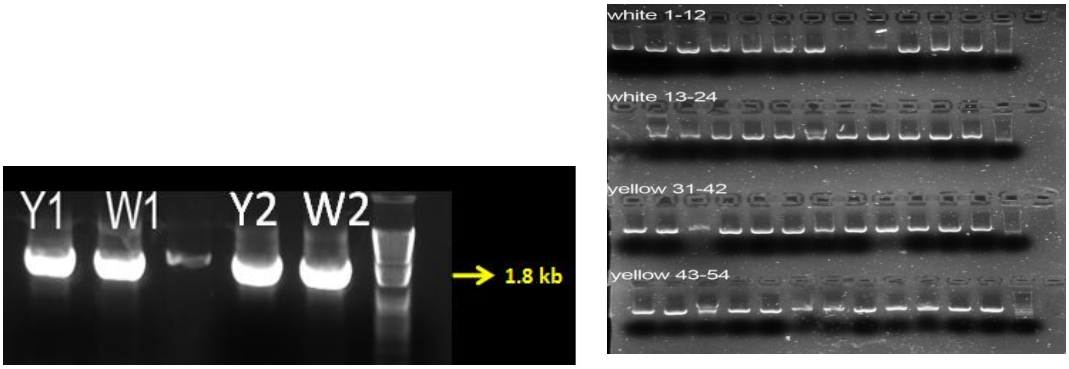
The predicted amino acid sequences of BoCCD4aW, BoCCD4aY, BoCCD4bW and BoCCD4bY consisted of 590, 590, 594 and 594 amino acids, respectively (Figure 7a). The four sequences were observed similar with variation in one or two amino acids between CCD4a and CCD4b irrespective of the petal colors (Figure 7a, b). The fast plant CCD4a proteins of white and yellow colors (BoCCD4aW & BoCCD4aY) showed 93.7 % identity whereas CCD4b proteins (BoCCD4bW & BoCCD4bY) displayed 99.5% identity to each other (Figure 7b). The proteins BoCCD4aW and BoCCD4bW from white phenotype showed 88.0% identity whereas BoCCD4aY and BoCCD4bY from yellow petals exhibited 82.0%

identity (Figure 7b). All the four amino acid sequences were compared with its close relative, cauliflower. The sequences BoCCD4aW and BoCCD4aY showed 98.8% and 92.7% identity with cauliflower CCD4a. Similarly, BoCCD4bW and BoCCD4bY displayed 96.1% and 95.7% sequence identity with cauliflower CCD4b (Figure 7b). Phylogenetic tree analysis indicated homology between fast plant CCD4 and cauliflower CCD4. Hence, the phylogeny based on amino acid substitutions were found with single amino acid variation in the whole sequence length (Figure 7b).

The first few amino acids of the four aligned sequences were predicted to be a chloroplast signal peptide in its N-terminal, suggesting that the plastidial localized CCD4 exhibited strong similarity between the two flower color types (Figure 7a). Also, the iron- ligating histidine (H) residues fixed by conserved aspartate or glutamate (Kloer and Schulz, 2006) were indicated in the protein sequences (Figure 7a). The obtained amino acid sequence profiles of fast plant phenotypes were compared with a similar data produced by Dr. Maria Laura, Agriaquaculture Nutritional Genomics Center (CGNA). The sequences BoCCD4a TO1000 (white), BoCCD4a Early big (yellow), BoCCD4b TO1000 (white) and BoCCD4b Early big (yellow) consisted of 593, 596, 593 and 455 amino acids, respectively. Of the four sequences, BoCCD4b Early big (yellow) was found distinct and unique from others. Other sequences revealed a strong similarity with AtCCD4 unlike BoCCD4b Early big type (Figure 7c).

The CCD4 sequences identified in this study (Figure 7a) were found almost similar to the provided data (Figure 7c) except for the variation observed between BoCCD4b Early big (yellow) and BoCCD4bY. The BoCCD4b Early big allele contained deletions in strings of sequences approximately at $380^{th}$ and $423^{rd}$ positions (Figure 7c) whereas such deletions were not observed in BoCCD4bY (Figure 7a). Although the chloroplast signal peptide was present in all four sequences, the peptide for BoCCD4b Early big was not aligned with the other three

and AtCCD4 (Figure 7c). Also, BoCCD4b Early big lacked a histidine residue at the 582$^{nd}$ position of the amino acid sequence in alignment with AtCCD4 (Figure 7c). The protein BoCCD4aY between 554 and 590 positions differed in its amino acid residues from other three proteins (Figure 7a). Lack of histidine was observed in 584$^{th}$ bp position of BoCCD4aY but not in BoCCD4bY (Figure 7a). Hence, amino acid sequences between BoCCD4b Early big & BoCCD4bY and BoCCD4a Early big & BoCCD4aY from figures 7a and 7c showed a wide variation.




**A** **B**

**Figure 5: PCR amplification and cloning *CCD4* from white and yellow fast plants. (A)** Amplification of *CCD4* with 1.8 kb product size. **(B)** Colony PCR for positive clones containing *CCD4* insert after *E.coli* competent cell transformation.

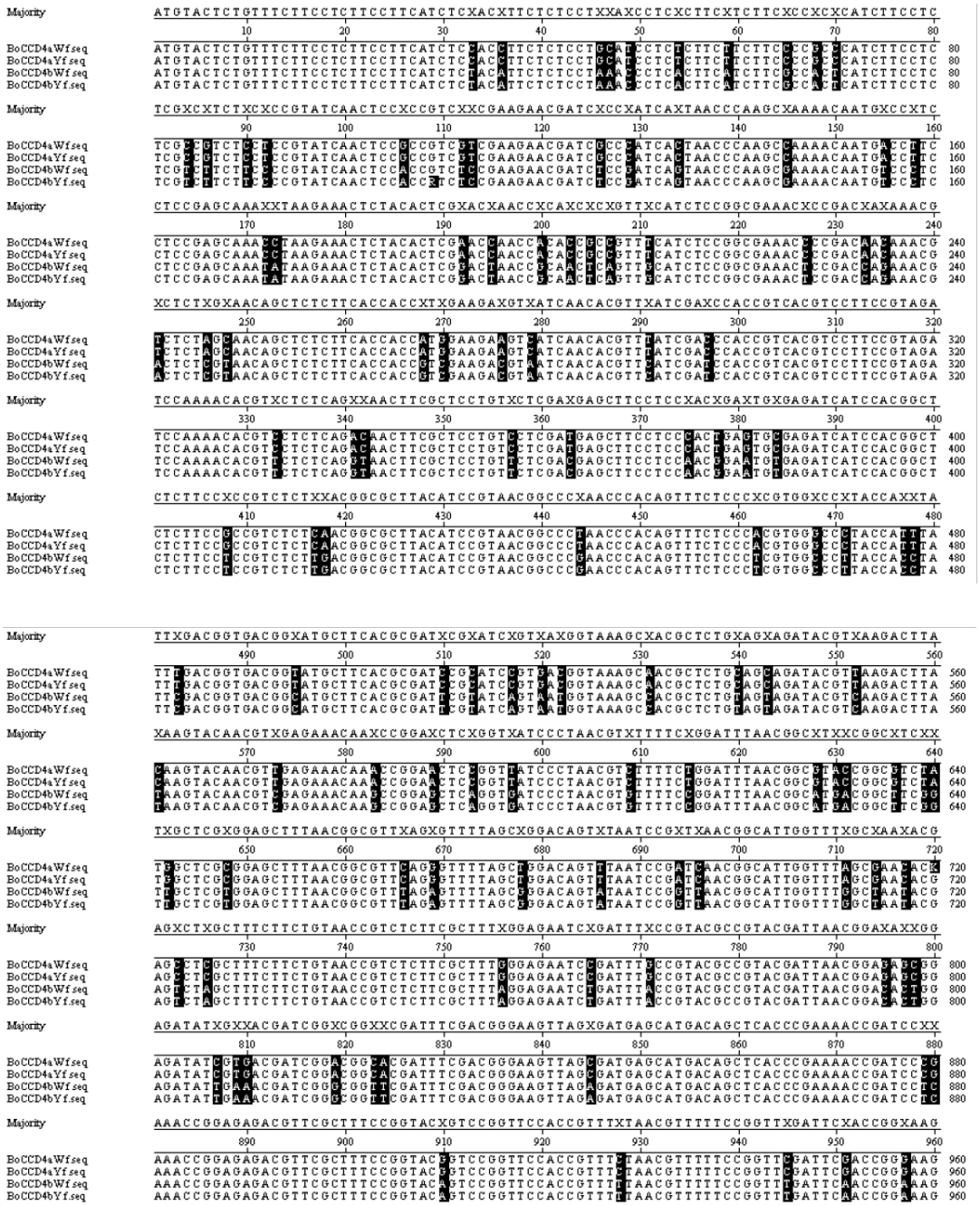
**Table 6a: Sequenced clones of fast plant phenotypes**

| Flower color | Clone number |
|---|---|
| White | 1, 2, 3, 4, 5, 6, 7, 10, 11,12,14,15,16,18,19, 20, 22, 23 |
| Yellow | 31, 32, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 47, 50, 53, 54 |

**Table 6b: Assembled contig groups from sequenced clones of white and yellow fast plants for the genes *CCD4a* and *CCD4b***

| Consensus name | Aligned clones | Given gene symbol |
|---|---|---|
| White group1 consensus | 19,23,10 | *BoCCD4bW* |
| White group2 consensus | 5,12,15,11,1,6,18, 2, 7, 22,16, 4, 20 | *BoCCD4aW* |
| Yellow group 2 consensus | 31,42,47,50,32 | *BoCCD4aY* |
| Yellow group 1 consensus | 35,37,41,43,44 | *BoCCD4bY* |

*gene symbol given in *Italics*. Clones that did not align with the above four groups were eliminated due to major nucleotide differences. PCR errors in the clones were corrected, sequences aligned and grouped.


Majority
BoCCD4aWf.seq
BoCCD4aYf.seq
BoCCD4bWf.seq
BoCCD4bYf.seq

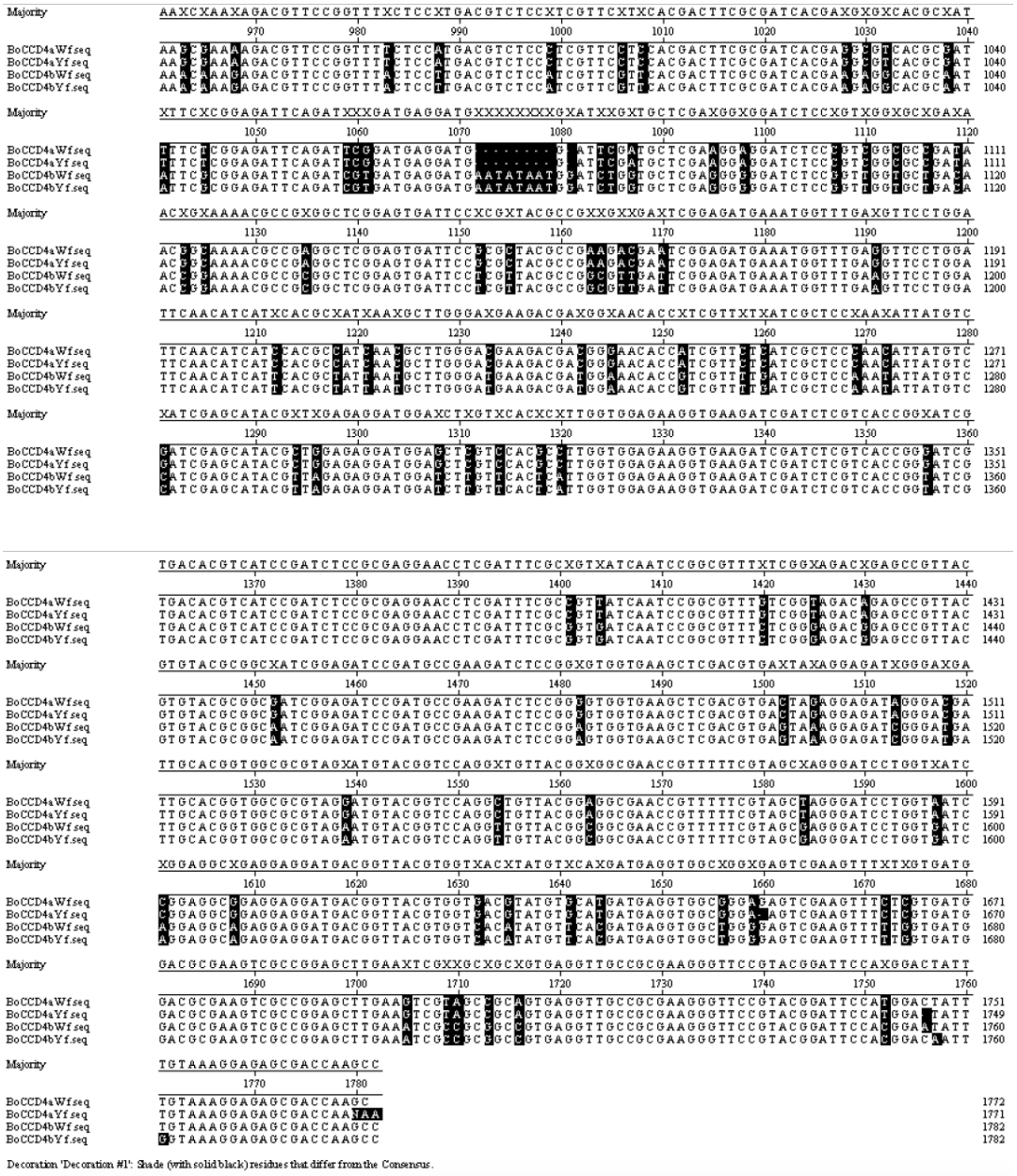



**Figure 6a: Nucleotide sequence alignment of fast plant genes *CCD4a* and *CCD4b* using ClustalW.** Fragment sizes of all four nucleotide sequences (*BoCCD4aW*, *BoCCD4a*Y, *BoCCD4bW*, and *BoCCD4bY*) are approximately 1.8 kb. The molecular polymorphism highlighted in dark between fast plant *CCD4a* and *CCD4b* sequences from both the petal colors show nucleotide variation.

**A.**

Percent Identity

| | 1 | 2 | 3 | 4 | | |
|---|---|---|---|---|---|---|
| 1 | | 99.8 | 88.0 | 87.9 | 1 | BoCCD4aW f.seq |
| 2 | 0.1 | | 87.9 | 87.7 | 2 | BoCCD4aY f.seq |
| 3 | 13.1 | 13.2 | | 99.8 | 3 | BoCCD4bW f.seq |
| 4 | 13.2 | 13.3 | 0.2 | | 4 | BoCCD4bY f.seq |
| | 1 | 2 | 3 | 4 | | |

Divergence

**B.**

Percent Identity

| | 1 | 2 | 3 | 4 | 5 | 6 | | |
|---|---|---|---|---|---|---|---|---|
| 1 | | 98.4 | 98.5 | 86.9 | 87.3 | 87.2 | 1 | Brassica oleracea CCD4a.seq |
| 2 | 1.5 | | 99.8 | 87.2 | 88.0 | 87.9 | 2 | BoCCD4aW f.seq |
| 3 | 1.5 | 0.1 | | 87.3 | 87.9 | 87.7 | 3 | BoCCD4aY f.seq |
| 4 | 14.5 | 14.0 | 13.9 | | 97.2 | 97.1 | 4 | Brassica oleracea CCD4b.seq |
| 5 | 14.0 | 13.1 | 13.2 | 2.9 | | 99.8 | 5 | BoCCD4bW f.seq |
| 6 | 14.0 | 13.2 | 13.3 | 2.9 | 0.2 | | 6 | BoCCD4bY f.seq |
| | 1 | 2 | 3 | 4 | 5 | 6 | | |

Divergence

**C.**

Percent Identity

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 99.8 | 88.0 | 87.9 | 98.4 | 87.2 | 84.7 | 61.1 | 62.4 | 64.3 | 56.0 | 57.8 | 59.1 | 67.7 | 61.2 | 65.5 | 64.9 | 63.3 | 63.2 | 67.9 | 58.3 | 65.2 | 1 | BoCCD4aW f.seq |
| 2 | 0.1 | | 87.9 | 87.7 | 98.5 | 87.3 | 84.8 | 61.0 | 62.3 | 64.3 | 55.9 | 57.7 | 59.0 | 67.7 | 61.2 | 65.5 | 64.9 | 63.2 | 63.2 | 67.9 | 58.4 | 65.3 | 2 | BoCCD4aY f.seq |
| 3 | 13.1 | 13.2 | | 99.8 | 87.3 | 97.2 | 86.3 | 61.3 | 61.7 | 64.4 | 56.2 | 56.2 | 57.3 | 66.8 | 59.8 | 64.6 | 64.1 | 62.8 | 63.1 | 67.4 | 56.8 | 64.6 | 3 | BoCCD4bW f.seq |
| 4 | 13.2 | 13.3 | 0.2 | | 87.2 | 97.1 | 86.1 | 61.3 | 61.6 | 64.3 | 56.1 | 56.2 | 57.3 | 66.7 | 59.7 | 64.6 | 64.0 | 62.8 | 63.0 | 67.3 | 56.8 | 64.5 | 4 | BoCCD4bY f.seq |
| 5 | 1.5 | 1.5 | 14.0 | 14.0 | | 86.9 | 85.1 | 61.0 | 62.7 | 64.5 | 55.8 | 57.6 | 58.9 | 68.1 | 61.2 | 65.7 | 65.7 | 63.2 | 63.7 | 68.2 | 58.7 | 65.3 | 5 | Brassica oleracea CCD4a.seq |
| 6 | 14.0 | 13.9 | 2.9 | 2.9 | 14.5 | | 85.2 | 60.3 | 60.8 | 63.8 | 54.8 | 56.1 | 57.2 | 66.0 | 59.6 | 64.5 | 64.2 | 62.6 | 62.2 | 66.8 | 55.7 | 64.3 | 6 | Brassica oleracea CCD4b.seq |
| 7 | 17.2 | 17.1 | 15.2 | 15.3 | 16.7 | 16.6 | | 62.0 | 60.6 | 64.6 | 57.1 | 56.0 | 57.1 | 62.1 | 56.1 | 64.5 | 64.4 | 64.4 | 61.1 | 62.8 | 57.6 | 65.5 | 7 | Arabidopsis thaliana.NCED4.seq |
| 8 | 55.9 | 56.0 | 55.3 | 55.2 | 56.1 | 57.5 | 54.0 | | 77.5 | 65.1 | 57.9 | 54.1 | 55.5 | 61.8 | 57.6 | 62.6 | 61.1 | 66.7 | 66.1 | 62.7 | 57.9 | 64.3 | 8 | Chrysanthemum x morifolium CCD4a.seq |
| 9 | 53.5 | 53.5 | 54.7 | 54.7 | 52.7 | 56.8 | 57.1 | 27.2 | | 68.2 | 57.2 | 54.9 | 56.2 | 61.1 | 56.6 | 64.4 | 64.0 | 68.0 | 67.7 | 61.7 | 60.1 | 66.7 | 9 | chrysanthemum x morifolium CCD4b.seq |
| 10 | 49.2 | 49.4 | 49.2 | 49.2 | 49.0 | 50.4 | 49.1 | 47.5 | 41.8 | | 60.0 | 57.5 | 58.6 | 67.6 | 59.7 | 69.4 | 68.2 | 68.0 | 69.1 | 69.0 | 59.4 | 71.9 | 10 | citrus clementina CCD4a.seq |
| 11 | 67.1 | 67.4 | 66.7 | 66.7 | 67.7 | 70.6 | 64.5 | 62.5 | 64.2 | 58.0 | | 52.6 | 53.2 | 55.7 | 54.1 | 57.0 | 57.0 | 58.5 | 58.2 | 56.5 | 54.6 | 58.2 | 11 | citrus clementina CCD4b.seq |
| 12 | 62.6 | 62.8 | 66.7 | 66.7 | 63.0 | 66.8 | 67.3 | 72.6 | 70.4 | 63.2 | 77.5 | | 99.5 | 62.6 | 66.1 | 58.1 | 57.1 | 55.1 | 53.9 | 62.4 | 55.9 | 57.4 | 12 | crocus sativus CCD4a.seq |
| 13 | 59.5 | 59.7 | 64.1 | 64.2 | 60.0 | 64.2 | 64.6 | 69.2 | 67.2 | 60.9 | 75.6 | 0.5 | | 63.4 | 67.2 | 58.7 | 58.1 | 56.2 | 55.3 | 63.3 | 56.5 | 58.4 | 13 | crocus sativus CCD4b.seq |
| 14 | 42.4 | 42.4 | 44.3 | 44.3 | 41.8 | 45.7 | 53.4 | 54.4 | 55.4 | 42.9 | 68.6 | 51.9 | 50.3 | | 58.8 | 71.8 | 68.0 | 65.6 | 62.1 | 86.9 | 61.9 | 69.3 | 14 | Fragaria vesca.seq |
| 15 | 54.9 | 55.1 | 58.3 | 58.3 | 55.0 | 58.6 | 66.4 | 63.9 | 65.9 | 58.7 | 72.5 | 45.6 | 43.7 | 60.1 | | 61.5 | 60.5 | 59.9 | 54.8 | 59.9 | 59.2 | 61.8 | 15 | Lilium brownii-CCD4.seq |
| 16 | 46.7 | 46.7 | 48.5 | 48.5 | 46.3 | 48.8 | 48.8 | 52.7 | 49.0 | 40.1 | 64.6 | 62.0 | 60.5 | 35.9 | 54.4 | | 69.6 | 66.7 | 66.2 | 73.1 | 61.2 | 71.4 | 16 | Malus x domestica CCD4.seq |
| 17 | 47.6 | 47.6 | 49.6 | 49.6 | 46.1 | 49.5 | 48.9 | 55.8 | 49.7 | 42.1 | 65.4 | 64.2 | 61.9 | 42.0 | 56.5 | 39.5 | | 65.7 | 64.9 | 69.7 | 60.4 | 68.1 | 17 | Momordica charantia CCD4.seq |
| 18 | 51.2 | 51.3 | 52.1 | 52.0 | 51.4 | 52.4 | 49.0 | 44.3 | 42.1 | 42.2 | 61.1 | 69.5 | 66.9 | 46.7 | 58.4 | 44.7 | 46.3 | | 72.2 | 66.5 | 60.4 | 69.2 | 18 | Nicotiana tabacum CCD4.seq |
| 19 | 51.9 | 51.9 | 52.1 | 52.1 | 50.9 | 53.9 | 55.7 | 45.7 | 42.8 | 40.3 | 62.3 | 72.9 | 69.5 | 53.6 | 70.1 | 45.7 | 48.2 | 35.3 | | 63.3 | 60.4 | 69.1 | 19 | Osmanthus fragrans CCD4.seq |
| 20 | 42.1 | 42.1 | 43.2 | 43.2 | 41.7 | 44.2 | 51.7 | 52.6 | 54.3 | 40.5 | 66.3 | 52.2 | 50.5 | 14.5 | 57.5 | 33.7 | 39.2 | 45.1 | 51.0 | | 61.7 | 71.0 | 20 | Rose CCD4.seq |
| 21 | 61.2 | 61.1 | 64.9 | 64.9 | 60.4 | 67.6 | 63.1 | 62.3 | 57.3 | 59.2 | 70.6 | 66.9 | 65.6 | 53.5 | 59.4 | 55.1 | 56.7 | 57.8 | 57.6 | 53.9 | | 61.9 | 21 | Scutellaria baicalensis CCD4.seq |
| 22 | 47.4 | 47.3 | 48.8 | 48.8 | 47.3 | 49.3 | 46.9 | 49.2 | 44.7 | 35.6 | 62.3 | 63.6 | 61.5 | 39.7 | 54.1 | 36.4 | 41.9 | 40.1 | 40.3 | 36.9 | 53.7 | | 22 | Yitis vinifera CCD4a.seq |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | | |

Divergence

**D.**

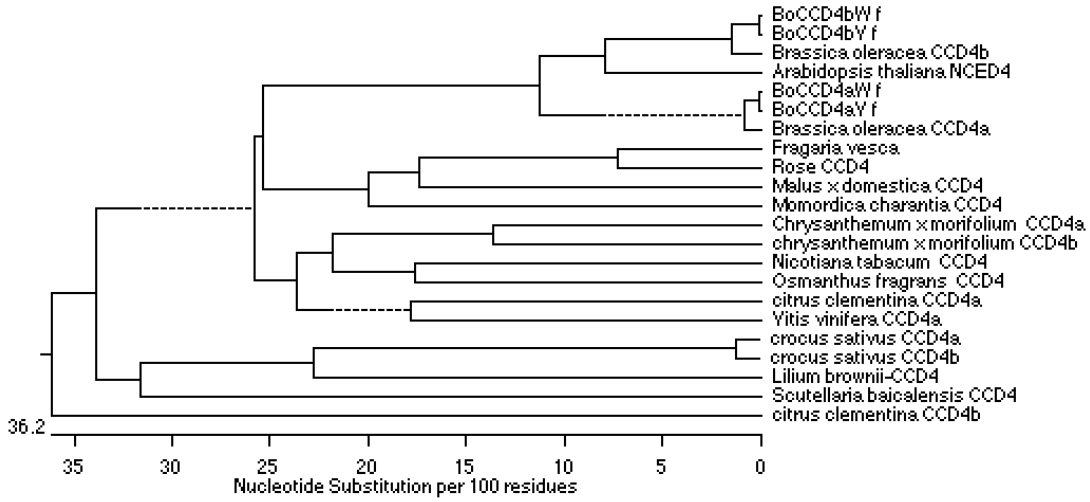



**Figure 6b: Sequence comparison of *CCD4*s between Brassica (fast plant) and other plant species. (A)** Sequence distances (% identity and divergence) between all four *CCD4* sequences (*BoCCD4aW*, *BoCCD4aY*, *BoCCD4bW*, and *BoCCD4bY*) of fast plant white and yellow flower colors. **(B), (C)** Comparison of sequence identity with cauliflower, its closest relative, and other plant *CCD4s*. **(D)** Phylogenetic tree analysis of predicted nucleotide sequences of plant *CCD4* genes based on sequence similarity. Only full-length coding sequences of the family members were included. Accession numbers are mentioned in **Table 7**.

**Table 7: List of *CCD4* accession numbers, sequence lengths and database sources for plant species used in comparison with fast plant *CCD4* isoforms**

| Species Name | Gene symbol | GenBank Accession number | Coding sequence length (kb) | Database source |
|---|---|---|---|---|
| *Arabidopsis thaliana* | *AtNCED4* | NM_118036.2 | 2.072 | NCBI |
| *Brassica oleracea (cauliflower)* | *BoCCD4a* | Bol009345 | 1.779 | Brassica |
| *Brassica oleracea (cauliflower)* | *BoCCD4b* | Bol029878 | 1.632 | Brassica |
| *Chrysanthemum x morifolium* | *CmCCD4a* | EU334432.1 | 1.800 | NCBI |
| *Chrysanthemumx morifolium* | *CmCCD4b* | AB247160.2 | 1.916 | NCBI |
| *Nicotiana tabacum* | *NtCCD4* | JF947192.1 | 1.806 | NCBI |
| *Citrus clementine* | *CcCCD4a* | DQ309330.1 | 1.812 | NCBI |
| *Citrus clementine* | *CcCCD4b* | DQ309331.1 | 1.683 | NCBI |
| *Crocus sativus* | *CsCCD4a* | EU523662.1 | 1.785 | NCBI |
| *Crocus sativus* | *CsCCD4b* | EU523663.1 | 1.710 | NCBI |
| *Vitis Vinifera* | *VvCCD4a* | JQ712827.1 | 1.799 | NCBI |
| *Malus x domestica* | *VvCCD4* | EU327777.1 | 1.692 | NCBI |
| *Fragaria vesca* | *FvCCD4* | XM_004297596.1 | 2.023 | NCBI |
| *Osmanthus fragrans* | *OfCCD4* | EU334434.1 | 2.077 | NCBI |
| *Rosa x damascena* | *RdCCD4* | EU334433.1 | 1.956 | NCBI |
| *Lilium brownie* | *LbCCD4* | AB733097.1 | 2.149 | NCBI |
| *Scutellaria baicalensis* | *SbCCD4* | KC760148.1 | 1.794 | NCBI |
| *Momordica charantia* | *McCCD4* | JX069769.1 | 1.791 | NCBI |

(NCBI- http://www.ncbi.nlm.nih.gov; Brassica - http://brassicadb.org)

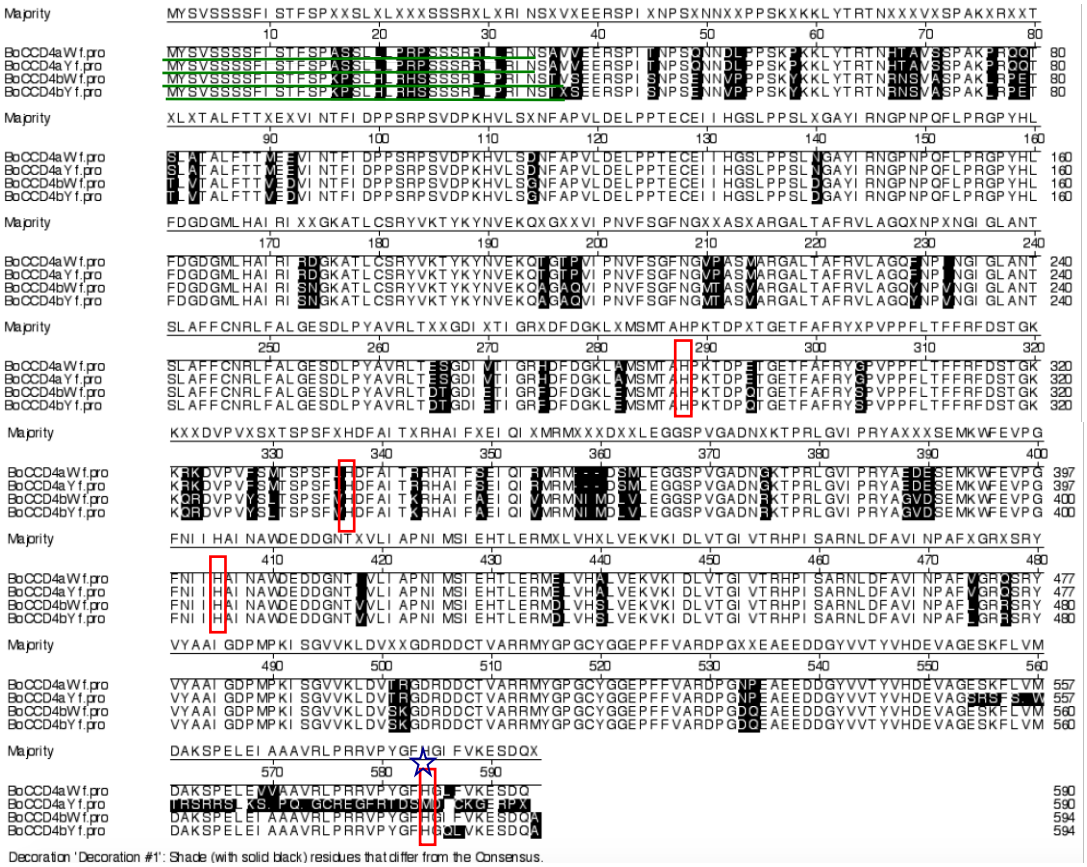



**Figure 7a: Amino acid sequence alignment of fast plant CCD4a and CCD4b using BLOSUM62 matrix with ClustalW.** Sequence lengths of BoCCD4aW, BoCCD4aY, BoCCD4bW and BoCCD4bY are 590, 590, 594 and 594 amino acids, respectively. The molecular polymorphism between CCD4a and CCD4b of the same or between the flower colors show amino acid variation. *green box indicates the chloroplast signal peptide.*red box indicates histidine residues. *star indicates absence of one of the histidines in BoCCD4aY.

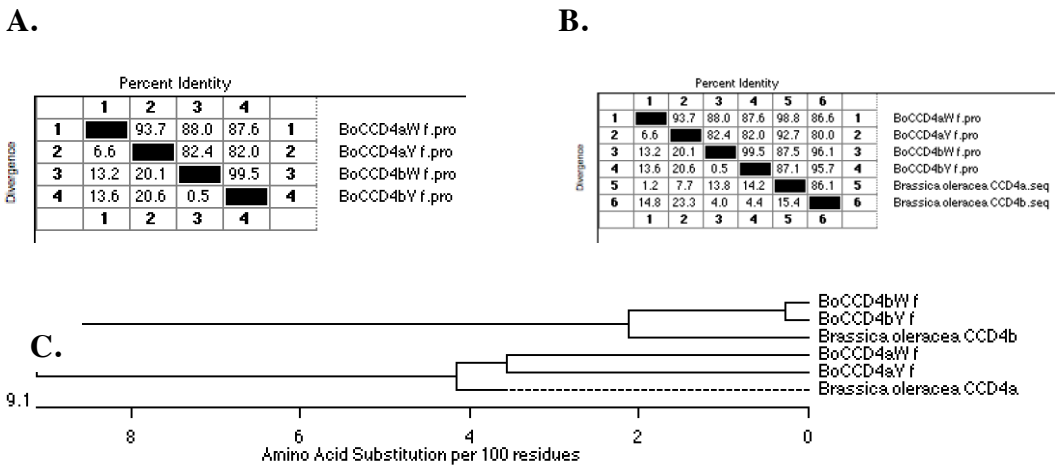



**Figure 7b: Amino acid sequence identity and phylogenetic tree analysis of CCD4 isoforms. (A)** Sequence distances (% identity and divergence) between all four CCD4 sequences (BoCCD4aW, BoCCD4aY, BoCCD4bW and BoCCD4bY) of fast plant phenotypes (White and Yellow). **(B)** Comparison of CCD4 sequence distances with cauliflower**.(C) P**hylogenetic tree analysis of deduced amino acid CCD4 sequences with its close relative *Brassica oleracea* (cauliflower). Only full-length coding sequences of the family members were included.

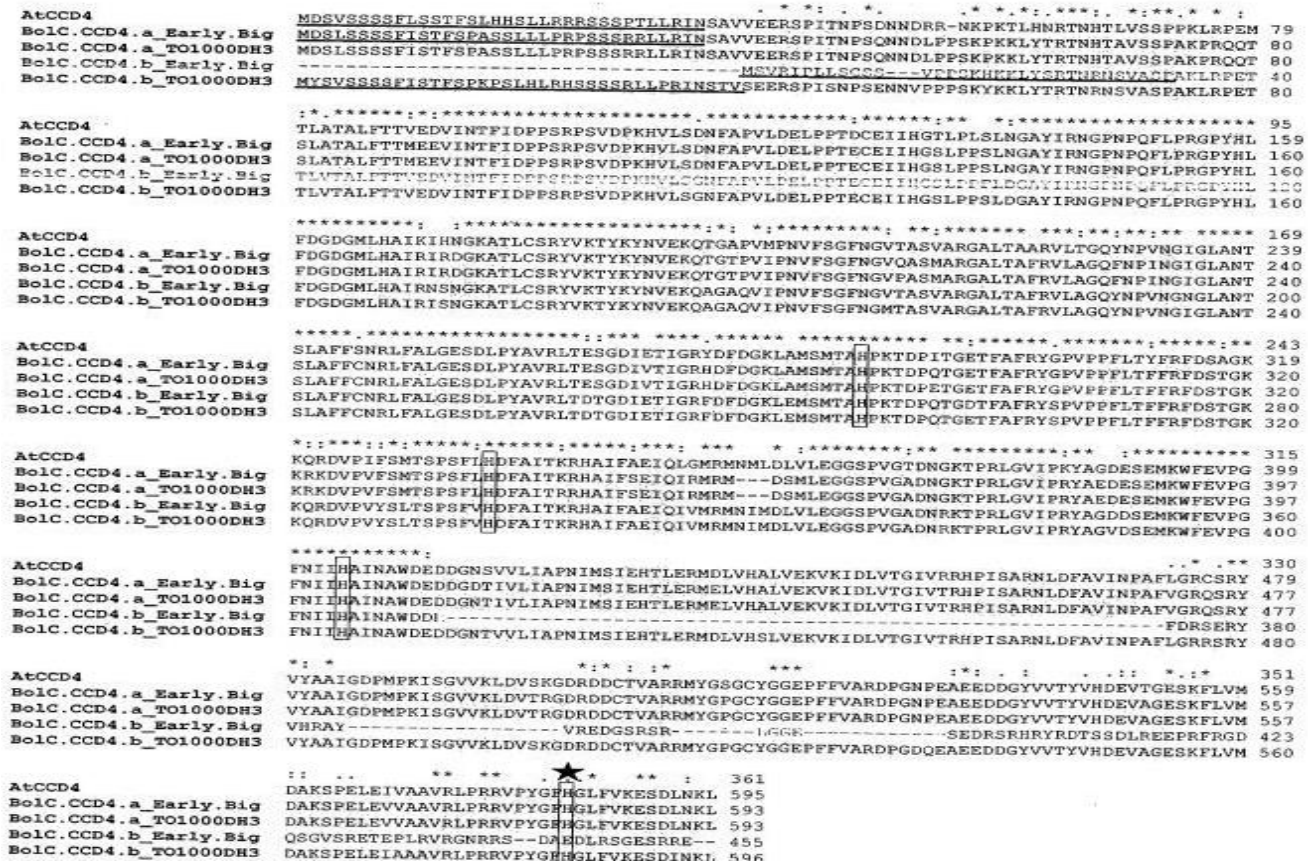



**Figure 7c: Multiple sequence alignment of predicted amino acids of *CCD4* alleles from fast plant flower color types in comparison with Arabidopsis CCD4 (AtCCD4).** Courtesy: data provided by Dr. Maria Laura, Agriaquaculture Nutritional Genomic Centre(CGNA). * underlined sequences indicate chloroplast sinal peptide. *box indicates histidine residues.★indicates absence of one of histidines in BoCCD4b Early Big allele.

### 3.4. Identification of fast plant *CCD4* promoter

The fast plant *CCD4a* and *CCD4b* coding sequences from different petal color varieties showed a few SNPs due to their high degree of similarity between them (Figure 6a). The aim was to identify markers for *CCD4* and to deduce its co-segregation with the flower color phenotype using $F_2$ population. Variation in the promoter sequence was predicted to be the major difference in mRNA expression levels observed in white and yellow petals for the *CCD4b* (Figure 4). Genome walking procedure was carried out to obtain the promoter sequence. The experiment failed as the obtained sequence (~ 400 bp) did not match with *CCD4* orthologs when performed NCBI-BLAST. Alternatively, a 2.0 kb *CCD4b* TO1000 (white) promoter sequence was identified from Brassica scaffold available in the database. Primers were designed from the white *CCD4b* promoter and *BoCCD4bY* (Table 2) so that the promoter sequence could be amplified from *CCD4b* yellow and compared with the white for any polymorphisms. The experiment did not succeed as the expected PCR product was not achieved after repeated attempts. If *CCD4b* promoter had shown a variation between the flower colors, molecular markers could have been developed to predict the association between the gene and the phenotype.

### 3.5. Functional analysis of *CCD4* alleles

Functional suppression of *CmCCD4a* transcription by means of RNA interference (RNAi) proved to modify white ray petals of chrysanthemum into yellow color thus responsible for petal coloration (Ohmiya *et al*.,2006*;* 2009). Based on qRT-PCR results (Table 5; Figure 4), the fast plant *BoCCD4b* was predicted to be the potential candidate in flower color modification. Hence, RNAi (knock-out) construct was designed and introduced a 1.1 kb fragment of *CCD4b* from yellow flower phenotype (Figure 2) into a plant vector,

pCAMBIA1300S. Future experiments on Agrobacterium mediated transformation might prove the functional role of *CCD4b* in fast plants.

Fast plant *CCD4* genes were functionally validated through recombinant expression in *E.coli*. β-carotene is one of the major substrates utilized by the enzyme CCD4 that preferentially cleaves at 9, 10 (9'10') double bond positions in plant species (Rubio *et al*., 2008; Huang *et al*., 2009b). The *E.coli* cells were introduced with plasmid pAC-Beta (Cunningham *et al*., 1996) and were co-transformed with CCD4 constructs developed in the study and the pET-Blue-1 carrying fast plant CCD4 constructs (provided by Dr Maria Laura). *E.coli* expressing these constructs were subjected to carotenoid extraction and quantitatively measured by HPLC method for the loss in orange color due to β-carotene degradation.

HPLC analyses was initially performed with BoCCD4aW, BoCCD4aY, BoCCD4bW and BoCCD4bY *E.coli* constructs. Beta-carotene degradation was not observed in any of the constructs. Hence, HPLC analyses was later performed for pET-Blue1 constructs which showed a loss in orange-colored beta-carotene over 50 % in *E. coli* carrying CCD4a Early big (yellow) and CCD4b TO1000 (white) compared to CCD4a TO1000 (white) and CCD4b Early big (yellow). The β-carotene peak was detected at wavelength 450 nm and 14 min retention time in all sample extracts and obtained the peak area. The control cell (pAC-Beta only) extract also led to loss in orange color similar to sample extracts (Figure 8). Similarly, the release of volatile β-ionone, a break-down product of β-carotene (Misawa *et al*., 1995) was detected in the bacterial headspace by SPME-GC-MS analyses using cultures produced from β-carotene producing *E.coli* carrying *CCD4* genes. The β-ionone elution due to CCD4 over-expression in *E.coli* harbouring pAC-Beta cells was calculated based on the abundance (peak integration) of the ion trace. The retention time at 26.92 min and mass spectrum at 177 m/z was detected in the external standard led to the identification of β-ionone in the *E.coli* cultures.

The results revealed that the volatile β-ionone released slightly higher in β-carotene producing *E.coli* cells harbouring CCD4b TO1000 (white) to that of CCD4b Early big (yellow). To the contrast, β-carotene producing *E.coli* cells harbouring CCD4a Early big (yellow) was found to elute higher β-ionone than CCD4a TO1000 (white). The control cell (pAC –Beta only) culture also released β-ionone equivalent to CCD4b Early big (yellow) culture (Figure 9). It has been reported that a small amount of β-ionone was detected in the headspace of control cultures harbouring empty vector pGEX-4T1 due to autoxidation of β-carotene (Huang *et al*., 2009b).

Previous studies in chrysanthemum reported that *CmCCD4a* contributes to white petal coloration (Ohmiya *et al*., 2006). High concentration of β-ionone was detected in the headspace of *E.coli* cells expressed with *CmCCD4a* upon cleavage at 9, 10 (9'10') positions (Huang *et al*., 2009b). Similarly, *CsCCD4* isolated from *Crocus sativus* exhibit β-carotene cleavage activity at 9, 10 (9'10') double bonds (Rubio *et al*., 2008). Other plant *CCD4*s from rose, osmanthus and apple preferentially cleave at 9, 10 (9', 10') double bonds irrespective of the substrates. All *CCD4* genes are expressed in flowers hence all CCD4 proteins produce β-ionone upon β-carotene cleavage except AtCCD4 and RdCCD4. The OfCCD4 has low cleavage activity (Huang *et al*., 2009b). From both HPLC and GC-MS analyses, it is predicted that the gene *CCD4b* may influence white flower color in fast plants due to β-carotene degradation and β-ionone release compared to gene *CCD4a*. The cleavage activity of CCD4a in fast plant phenotypes appeared partial or low which predicted that CCD4a may not accept β-carotene similar to AtCCD4 and RdCCD4. Although, β-ionone release was observed in CCD4b TO1000 (white) and CCD4b Early big (yellow) *E.coli* cultures, the difference was not phenomenal (Figure 9). Also, the β-carotene in cell extracts was not completely degraded when analysed by HPLC (Figure 8). Thus, the cleavage activity of CCD4b in fast plants appeared not clear which may be due to (i) preference for different substrates at different positions (Lashbrooke *et al*., 2013; Bouvier *et al*., 2003; Rubio *et al*., 2008) (ii) no role in

carotenoid degradation although the expression pattern was found flower-specific (Figure 4) and intron-less (Figure 6a). In this context, *CmCCD4a* and *MdCCD4* are intron-rich, expressed flower-specific and substrate specific to β-carotene. The intron-less *AtCCD4* and *RdCC4* exhibit similar substrate specificity in their genomic DNA but preferentially cleave a different substrate, $C_{30}$ apocarotenoid. The *OfCCD4* (*Osmanthus fragrans*) contain introns and its expression is specific to flowers but enzyme does not cleave both carotenoids and apocarotenoids well (Huang *et al*., 2009b). The expression pattern of CCD4 in various plant species, its intron number and substrate specific cleavage determined that CCD4 ancestral gene evolved into new allelic forms with distinct properties (Huang *et al*., 2009b; Ahrazem *et al*., 2010). Although CCD4 is expressed in flowers of *Ipomoea sp*., there is no role in carotenoid breakdown (Yamamizo *et al*., 2010). Similarly, the biological role of RdCCD4 (*Rosa damascena*) is still unclear though its expression is confined to flowers (Huang *et al*., 2009b). This suggests that fast plant CCD4 may have a different role from petal coloration regardless of its flower-specific expression. However, genetic studies in peach proved that *PpCCD4* as the *Y* gene controlling fruit flesh color (Adami *et al*., 2013). Hence, molecular markers for *CCD4a* and *CCD4b* might prove its association with flower petal color in fast plants.

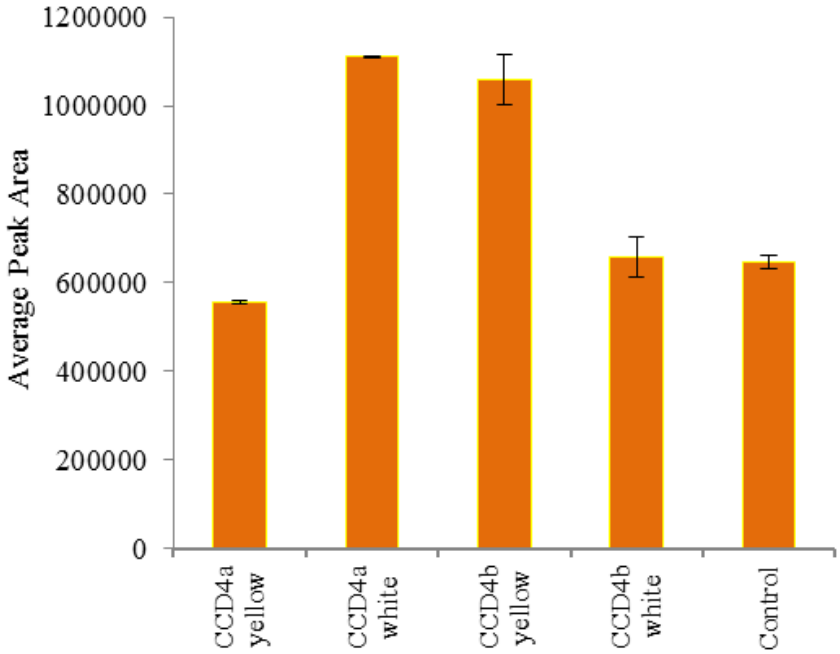
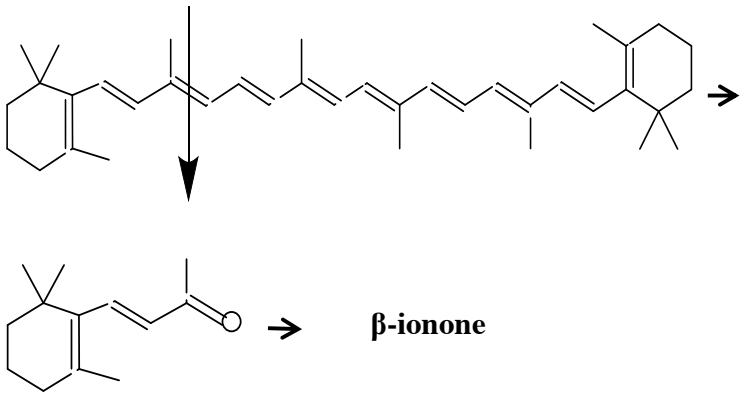
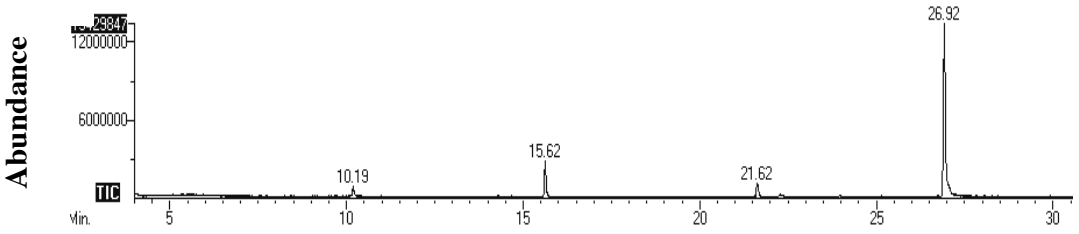



**Figure 8: Functional analyses of fast plant CCD4 *E.coli* cells harboring pAC-Beta producing β-carotene.** HPLC analysis of β-carotene degradation in CCD4 expressing cells based on average peak area at 450 nm wavelength and 14 min retention time (Y-axis).

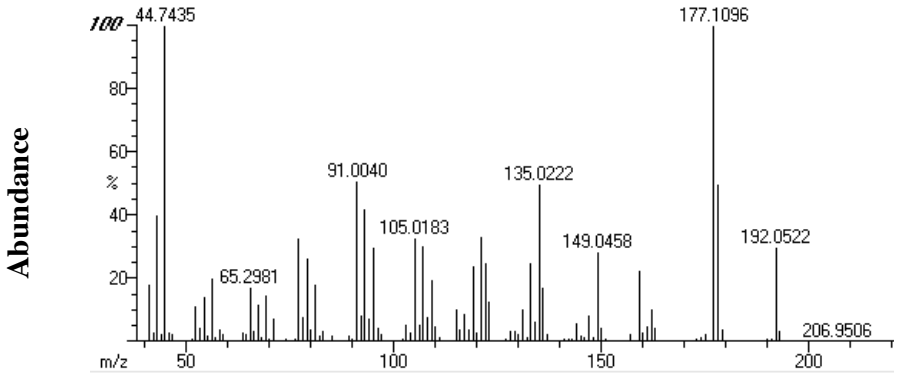
**A.**




**B.**




**Time (min)**




**m/z**

**C.**

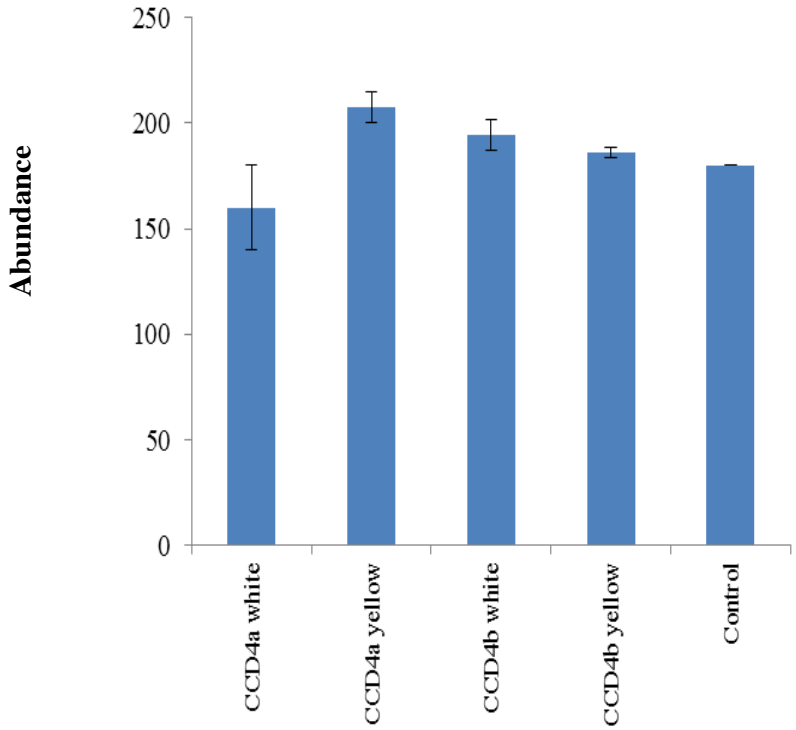



**Figure 9: Functional analyses of fast plant CCD4 E.coli cells harboring pAC-Beta producing β-carotene through GC-MS method. (A)** The volatile β-ionone upon β-carotene cleavage at 9,10 (9'10') double bond positions **(B)** Standard chromatogram at 26.92 min retention time and mass spectra at 177 m/z **(C)** Graphical representation of GC-MS analyses of headspace volatile emission of β-ionone from *E.coli* cells.

## REFERENCES


Adami, M., De Franceschi, P., Brandi, F., Liverani, A., Giovannini, D., Rosati, C., Dondini, L. & Tartarini, S. (2013). Identifying a carotenoid cleavage dioxygenase (*CCD4*) gene controlling yellow/white fruit flesh color of peach. *Plant Molecular Biology Reporter*, *31*(5), 1166-1175.

Ahrazem, O., Trapero, A., Gómez, M. D., Rubio-Moraga, A., & Gómez-Gómez, L. (2010). Genomic analysis and gene structure of the plant carotenoid dioxygenase 4 family: A deeper study in *Crocus sativus* and its allies. *Genomics*, *96*(4), 239-250.

Al-Babili, S., Hugueney, P., Schledz, M., Welsch, R., Frohnmeyer, H., Laule, O., & Beyer, P. (2000). Identification of a novel gene coding for neoxanthin synthase from *Solanum tuberosum*. *FEBS letters*, *485*(2), 168-172.

Ampomah-Dwamena, C., Dejnoprat, S., Lewis, D., Sutherland, P., Volz, R. K., & Allan, A. C. (2012). Metabolic and gene expression analysis of apple (*Malus× domestica*) carotenogenesis. *Journal of experimental botany*, 1-15.

Auldridge, M. E., Block, A., Vogel, J. T., Dabney-Smith, C., Mila, I., Bouzayen, Magallanes-Lundback, M., DellaPenna, D., McCarty, D.R. & Klee, H. J. (2006b). Characterization of three members of the Arabidopsis carotenoid cleavage dioxygenase family demonstrates the divergent roles of this multifunctional enzyme family. *The Plant Journal*, *45*(6), 982-993.

Auldridge, M. E., McCarty, D. R., & Klee, H. J. (2006a). Plant carotenoid cleavage oxygenases and their apocarotenoid products. *Current opinion in plant biology*, *9*(3), 315-321.

Bai, C., Twyman, R. M., Farré, G., Sanahuja, G., Christou, P., Capell, T., & Zhu, C. (2011). A golden era—pro-vitamin A enhancement in diverse crops. *In Vitro Cellular & Developmental Biology-Plant*, *47*(2), 205-221.

Bailey, S., & Grossman, A. (2008). Photoprotection in Cyanobacteria: Regulation of Light Harvesting†. *Photochemistry and Photobiology*, *84*(6), 1410-1420.

Baldermann, S., Kato, M., Kurosawa, M., Kurobayashi, Y., Fujita, A., Fleischmann, P., & Watanabe, N. (2010). Functional characterization of a carotenoid cleavage dioxygenase 1 and its relation to the carotenoid accumulation and volatile emission during the floral development of *Osmanthus fragrans* Lour. *Journal of experimental botany*, *61*(11), 2967-2977.

Booker, J., Auldridge, M., Wills, S., McCarty, D., Klee, H., & Leyser, O. (2004). MAX3/CCD7 is a carotenoid cleavage dioxygenase required for the synthesis of a novel plant signaling molecule. *Current biology*, *14*(14), 1232-1238.

Bouvier, F., D'Harlingue, A., Backhaus, R. A., Kumagai, M. H., & Camara, B. (2000). Identification of neoxanthin synthase as a carotenoid cyclase paralog. *European Journal of Biochemistry*, *267*(21), 6346-6352.

Bouvier, F., Isner, J. C., Dogbo, O., & Camara, B. (2005). Oxidative tailoring of carotenoids: a prospect towards novel functions in plants. *Trends in plant science*, *10*(4), 187-194.

Bouvier, F., Suire, C., Mutterer, J., & Camara, B. (2003). Oxidative Remodeling of Chromoplast Carotenoids Identification of the Carotenoid Dioxygenase CsCCD and CsZCD Genes Involved in Crocus Secondary Metabolite Biogenesis. *The Plant Cell Online*, *15*(1), 47-62.

Brandi, F., Bar, E., Mourgues, F., Horváth, G., Turcsi, E., Giuliano, G., Liverani, A., Tartarini, S., Lewinsohn, E. & Rosati, C. (2011). Study of 'Redhaven' peach and its white-fleshed mutant

suggests a key role of CCD4 carotenoid dioxygenase in carotenoid and norisoprenoid volatile metabolism. *BMC plant biology*, *11*(1), 24.

Bray, E. A. (2002). Abscisic acid regulation of gene expression during water-deficit stress in the era of the Arabidopsis genome. *Plant, cell & environment*, *25*(2), 153-161.

Britton, G., Liaaen-Jensen, S., & Pfander, H. (Eds.). (2004). *Carotenoids: handbook*. Springer.

Burbidge, A., Grieve, T. M., Jackson, A., Thompson, A., McCarty, D. R., & Taylor, I. B. (1999). Characterization of the ABA-deficient tomato mutantnotabilis and its relationship with maize Vp14. *The Plant Journal*, *17*(4), 427-431.

Campbell, R., Ducreux, L. J., Morris, W. L., Morris, J. A., Suttle, J. C., Ramsay, G., Bryan, G.J., Hedley, P.E. & Taylor, M. A. (2010). The metabolic and developmental roles of carotenoid cleavage dioxygenase4 from potato. *Plant physiology*, *154*(2), 656-664.

Cazzonelli, C. I., & Pogson, B. J. (2010). Source to sink: regulation of carotenoid biosynthesis in plants. *Trends in plant science*, *15*(5), 266-274.

Cazzonelli, C.I., Nisar, N., Hussain, D., Carmody, M.E., & Pogson, B.J. (2010). Biosynthesis and Regulation of Carotenoids in Plants—micronutrients, vitamins and health benefits. In Plant Developmental Biology-Biotechnology Perspectives (Vol. 2) Pua, E.C. and Davey, M.R., eds In pp. 117–137, Berlin Heidelberg, Springer-Verlag.

Chappell, J. (1995). The biochemistry and molecular biology of isoprenoid metabolism. *Plant physiology*, *107*(1), 1.

Chen, Y., Li, F., & Wurtzel, E. T. (2010). Isolation and characterization of the Z-ISO gene encoding a missing component of carotenoid biosynthesis in plants. *Plant Physiology*, *153*(1), 66-79.

Chernys, J. T., & Zeevaart, J. A. D. (2000). Characterization of the 9-cis-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in avocado. *Plant Physiology*, *124*(1), 343-354.

Cunningham Jr, F. X., & Gantt, E. (1998). Genes and enzymes of carotenoid biosynthesis in plants. *Annual review of plant biology*, 49(1), 557-583.

Cunningham Jr, F. X., Pogson, B., Sun, Z., McDonald, K. A., DellaPenna, D., & Gantt, E. (1996). Functional analysis of the β and ϵ lycopene cyclase enzymes of Arabidopsis reveals a mechanism for control of cyclic carotenoid formation. *The Plant Cell*, *8*, 1613-1626.

Cunningham, F. X., & Gantt, E. (2001). One ring or two? Determination of ring number in carotenoids by lycopene ε-cyclases. *Proceedings of the National Academy of Sciences*, *98*(5), 2905-2910.

Cuttriss, A.J., Cazzonelli, C.I., Wurtzel, E.T. & Pogson, B.J. (2011). "Carotenoids" In Biosynthesis of Vitamins in Plants. *Advances in Botanical Research*. (Vol.58) Rébeillé, F. and Douce, R., eds In pp. 1-36, Amsterdam Netherlands, Elsevier.

D'Ambrosio, C., Giorio, G., Marino, I., Merendino, A., Petrozza, A., Salfi, L., Stigliani, A. L. & Cellini, F. (2004). Virtually complete conversion of lycopene into β-carotene in fruits of tomato plants transformed with the tomato lycopene β-cyclase (lcy-b) cDNA. *Plant Science*, *166*(1), 207-214.

DellaPenna, D., & Pogson, B. J. (2006). Vitamin synthesis in plants: tocopherols and carotenoids. *Annual review of plant biology*, *57*, 711-738.

Demmig-Adams, B., & Adams III, W. W. (1996). The role of xanthophyll cycle carotenoids in the protection of photosynthesis. *Trends in plant science*, *1*(1), 21-26.

Demmig-Adams, B., & Adams, W. W. (2002). Antioxidants in photosynthesis and human nutrition. *Science*, *298*(5601), 2149-2153.

Dharmapuri, S., Rosati, C., Pallara, P., Aquilani, R., Bouvier, F., Camara, B., & Giuliano, G. (2002). Metabolic engineering of xanthophyll content in tomato fruits. *FEBS letters*, *519*(1), 30-34.

Diretto, G., Al-Babili, S., Tavazza, R., Papacchioli, V., Beyer, P., & Giuliano, G. (2007a). Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. *PLoS One*, *2*(4), e350.

Diretto, G., Tavazza, R., Welsch, R., Pizzichini, D., Mourgues, F., Papacchioli, V., Beyer, P. & Giuliano, G. (2006). Metabolic engineering of potato tuber carotenoids through tuber-specific silencing of lycopene epsilon cyclase. *BMC Plant Biology*, *6*(1), 13.

Diretto, G., Welsch, R., Tavazza, R., Mourgues, F., Pizzichini, D., Beyer, P., & Giuliano, G. (2007b). Silencing of beta-carotene hydroxylase increases total carotenoid and beta-carotene levels in potato tubers. *BMC plant biology*, *7*(1), 11.

Drummond, R. S., Martínez-Sánchez, N. M., Janssen, B. J., Templeton, K. R., Simons, J. L., Quinn, B. D., Karunairetnam, S. & Snowden, K. C. (2009). Petunia hybrida CAROTENOID CLEAVAGE DIOXYGENASE7 is involved in the production of negative and positive branching signals in petunia. *Plant physiology*, *151*(4), 1867-1877.

Ducreux, L. J., Morris, W. L., Hedley, P. E., Shepherd, T., Davies, H. V., Millam, S., & Taylor, M. A. (2005). Metabolic engineering of high carotenoid potato tubers containing enhanced levels of β-carotene and lutein. *Journal of experimental botany*, *56*(409), 81-89.

El-Agamey, A., Lowe, G. M., McGarvey, D. J., Mortensen, A., Phillip, D. M., Truscott, T. G., & Young, A. J. (2004). Carotenoid radical chemistry and antioxidant/pro-oxidant properties. *Archives of biochemistry and biophysics*, *430*(1), 37-48.

Farré, G., Sanahuja, G., Naqvi, S., Bai, C., Capell, T., Zhu, C., & Christou, P. (2010). Travel advice on the road to carotenoids in plants. *Plant Science*, *179*(1), 28-48.

Fraser, P. D., & Bramley, P. M. (2004). The biosynthesis and nutritional uses of carotenoids. *Progress in lipid research*, *43*(3), 228-265.

Fraser, P. D., Enfissi, E. M., Halket, J. M., Truesdale, M. R., Yu, D., Gerrish, C., & Bramley, P. M. (2007). Manipulation of phytoene levels in tomato fruit: effects on isoprenoids, plastids, and intermediary metabolism. *The Plant Cell Online*, *19*(10), 3194-3211.

Fraser, P. D., Enfissi, E., & Bramley, P. M. (2009). Genetic engineering of carotenoid formation in tomato fruit and the potential application of systems and synthetic biology approaches. *Archives of Biochemistry and Biophysics*, *483*(2), 196-204.

Frusciante, S., Diretto, G., Bruno, M., Ferrante, P., Pietrella, M., Prado-Cabrero, A., Rubio-Moraga, A., Beyer, P., Gomez-Gomez, L., Al-Babili, S. & Giuliano, G. (2014). Novel carotenoid cleavage dioxygenase catalyzes the first dedicated step in saffron crocin biosynthesis. *Proceedings of the National Academy of Sciences*, *111*(33), 12246-12251.

García-Limones, C., Schnäbele, K., Blanco-Portales, R., Luz Bellido, M., Caballero, J. L., Schwab, W., & Muñoz-Blanco, J. (2008). Functional characterization of FaCCD1: a carotenoid cleavage dioxygenase from strawberry involved in lutein degradation during fruit ripening. *Journal of Agricultural and food chemistry*, *56*(19), 9277-9285.

Giuliano, G. (2014). Plant carotenoids: genomics meets multi-gene engineering. *Current opinion in plant biology*, *19*, 111-117.

Gomez-Roldan, V., Fermas, S., Brewer, P. B., Puech-Pagès, V., Dun, E. A., Pillot, J. P., Letisse, F., Matusova, R., Danoun, S., Portais, J.C., Bouwmeester, H., Bécard, G., Beveridge C.A.,

Rameau, C. & Rochange, S. F. (2008). Strigolactone inhibition of shoot branching. *Nature*, *455*(7210), 189-194.

Gonzalez-Jorge, S., Ha, S. H., Magallanes-Lundback, M., Gilliland, L. U., Zhou, A., Lipka, A. E., Nhu-Nguyen, Y., Angelovici, R., Cepela, J., Little, H., Buell, C.R., Gore, M.A. & DellaPenna, D. (2013). CAROTENOID CLEAVAGE DIOXYGENASE4 Is a Negative Regulator of β-Carotene Content in Arabidopsis Seeds. *The Plant Cell Online*, *25*(12), 4812-4826.

Han, Y., Wang, X., Chen, W., Dong, M., Yuan, W., Liu, X., & Shang, F. (2014). Differential expression of carotenoid-related genes determines diversified carotenoid coloration in flower petal of *Osmanthus fragrans*. *Tree Genetics & Genomes*, *10*(2), 329-338.

Harjes, C.E., Rocheford, T.R., Bai, L., Brutnell, T.P., Kandianis, C.B., Sowinski, S.G., Stapleton, A.E., Vallabhaneni, R., Williams, M., Wurtzel, E.T.,Yan, J.B. & Buckler, E.S. (2008). Natural genetic variation in lycopene epsilon cyclase tapped for maize biofortification. *Science*, *319*, 330-333.

Harrison, E. H. (2005). Mechanisms of digestion and absorption of dietary Vitamin A*. *Annual review of nutrition*, *25*, 87-103.

Hattori, K. (1991). Inheritance of carotenoid pigmentation in flower color of chrysanthemum. *Japanese Journal of Breeding*, *41*(1), 1-10.

Hirschberg, J. (2001). Carotenoid biosynthesis in flowering plants. *Current opinion in plant biology*, *4*(3), 210-218.

Hirschi, K.D. (2009).Nutrient biofortification of food crops. *Annual Review of Nutrition*, *29*,401-421.

Howitt, C. A., & Pogson, B. J. (2006). Carotenoid accumulation and function in seeds and non-green tissues. *Plant, cell & environment*, *29*(3), 435-445.

Huang, F. C., Horváth, G., Molnár, P., Turcsi, E., Deli, J., Schrader, J., Sandmann, G., Schmidt, H. & Schwab, W. (2009a). Substrate promiscuity of RdCCD1, a carotenoid cleavage dioxygenase from *Rosa damascena*. *Phytochemistry*, *70*(4), 457-464.

Huang, F. C., Molnár, P., & Schwab, W. (2009b). Cloning and functional characterization of carotenoid cleavage dioxygenase 4 genes. *Journal of experimental botany*, erp137.

Huang, J. C., Zhong, Y. J., Liu, J., Sandmann, G., & Chen, F. (2013). Metabolic engineering of tomato for high-yield production of astaxanthin. *Metabolic engineering*, *17*, 59-67.

Ibdah, M., Azulay, Y., Portnoy, V., Wasserman, B., Bar, E., Meir, A., Burger, Y., Hirschberg, J., Schaffer, A.A., Katzir, N., Tadmor, Y. & Lewinsohn, E. (2006). Functional characterization of CmCCD1, a carotenoid cleavage dioxygenase from melon. *Phytochemistry*, *67*(15), 1579-1589.

Ilg, A., Beyer, P., & Al-Babili, S. (2009). Characterization of the rice carotenoid cleavage dioxygenase 1 reveals a novel route for geranial biosynthesis. *FEBS Journal*, *276*(3), 736-747.

Ilg, A., Yu, Q., Schaub, P., Beyer, P., & Al-Babili, S. (2010). Overexpression of the rice carotenoid cleavage dioxygenase 1 gene in Golden Rice endosperm suggests apocarotenoids as substrates in planta. *Planta*, *232*(3), 691-699.

IOM. (2001). Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc. *National Academy Press*. Washington, DC.

Isaacson, T., Ohad, I., Beyer, P., & Hirschberg, J. (2004). Analysis in vitro of the enzyme CRTISO establishes a poly-cis-carotenoid biosynthesis pathway in plants. *Plant physiology*, *136*(4), 4246-4255.

Isaacson, T., Ronen, G., Zamir, D., & Hirschberg, J. (2002). Cloning of tangerine from tomato reveals a carotenoid isomerase essential for the production of β-carotene and xanthophylls in plants. *The Plant Cell*, *14*(2), 333-342.

Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., Tabata, S., Kakubari, Y., Shinozaki, K.Y. & Shinozaki, K. (2001). Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. *The Plant Journal*, *27*(4), 325-333.

Iuchi, S., Kobayashi, M., Yamaguchi-Shinozaki, K., & Shinozaki, K. (2000). A stress-inducible gene for 9-cis-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. *Plant Physiology*, *123*(2), 553-562.

Johnson, X., Brcich, T., Dun, E. A., Goussot, M., Haurogné, K., Beveridge, C. A., & Rameau, C. (2006). Branching genes are conserved across species. Genes controlling a novel signal in pea are coregulated by other long-distance signals. *Plant physiology*, *142*(3), 1014-1026.

Kato, M., Matsumoto, H., Ikoma, Y., Okuda, H., & Yano, M. (2006). The role of carotenoid cleavage dioxygenases in the regulation of carotenoid profiles during maturation in citrus fruit. *Journal of Experimental Botany*, *57*(10), 2153-2164.

Kim, S. H., Ahn, Y. O., Ahn, M. J., Lee, H. S., & Kwak, S. S. (2012). Down-regulation of β-carotene hydroxylase increases β-carotene and total carotenoids enhancing salt stress tolerance in transgenic cultured cells of sweet potato. *Phytochemistry*, *74*, 69-78.

Kishimoto, S., & Ohmiya, A. (2006). Regulation of carotenoid biosynthesis in petals and leaves of chrysanthemum (*Chrysanthemum morifolium*). *Physiologia Plantarum*, *128*(3), 436-447.

Kishimoto, S., Maoka, T., Nakayama, M., & Ohmiya, A. (2004). Carotenoid composition in petals of chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura). *Phytochemistry*, *65*(20), 2781-2787.

Kishimoto, S., Sumitomo, K., Yagi, M., Nakayama, M., & Ohmiya, A. (2007). Three routes to orange petal color via carotenoid components in 9 Compositae species. *Journal of the Japanese Society for Horticultural Science*, *76*(3), 250-257.

Kloer, D. P., & Schulz, G. E. (2006). Structural and biological aspects of carotenoid cleavage. *Cellular and Molecular Life Sciences CMLS*, *63*(19-20), 2291-2303.

Krinsky, N. I., Landrum, J. T., & Bone, R. A. (2003). Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. *Annual review of nutrition*, *23*(1), 171-201.

Lashbrooke, J. G., Young, P. R., Dockrall, S. J., Vasanth, K., & Vivier, M. A. (2013). Functional characterisation of three members of the *Vitis vinifera* L. carotenoid cleavage dioxygenase gene family. *BMC plant biology*, *13*(1), 156.

Li, F. Q., Murillo, C. and Wurtzel, E. T. (2007). Maize Y9 encodes a product essential for 15-cis-zeta-carotene isomerization. *Plant Physiology*, *144*, 1181–1189.

Li, F., Vallabhaneni, R., & Wurtzel, E. T. (2008). PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and regulator of abiotic stress-induced root carotenogenesis. *Plant Physiology*, *146*(3), 1333-1345.

Li, L., & Van Eck, J. (2007). Metabolic engineering of carotenoid accumulation by creating a metabolic sink. *Transgenic research*, *16*(5), 581-585.

Li, L., Paolillo, D. J., Parthasarathy, M. V., DiMuzio, E. M., & Garvin, D. F. (2001). A novel gene mutation that confers abnormal patterns of β-carotene accumulation in cauliflower (*Brassica oleracea* var. *botrytis*). *The Plant Journal*, *26*(1), 59-67.

Li, L., Yang, Y., Xu, Q., Owsiany, K., Welsch, R., Chitchumroonchokchai, C., Lu, S., Van Eck, J., Xin Deng, X., Failla, M. & Thannhauser, T. W. (2012). The *Or* gene enhances carotenoid accumulation and stability during post-harvest storage of potato tubers. *Molecular plant*, *5*(2), 339-352.

Li, Q., Farre, G., Naqvi, S. Breitenbach, J. Sanahuja, G., Bai, C., Sandmann, G., Capell, T., Christou, P. and Zhu, C. (2010). Cloning and functional characterization of the maize carotenoid isomerase and β-carotene hydroxylase genes and their regulation during endosperm maturation. *Transgenic Research*, *19*, 1053–1068.

Lopez, A. B., Van Eck, J., Conlin, B. J., Paolillo, D. J., O'Neill, J., & Li, L. (2008). Effect of the cauliflower *Or* transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *Journal of experimental botany*, *59*(2), 213-223.

Lu, S., Van Eck, J., Zhou, X., Lopez, A. B., O'Halloran, D. M., Cosman, K. M., Brian, J.C., Paolillo, D.J., Garvin, D.F., Vrebalov, J., Kochian, L.V., Kupper, H., Earle, E.D., Cao, J. & Li, L. (2006). The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of β-carotene accumulation. *The Plant Cell Online*, *18*(12), 3594-3605.

Lyi, S. M., Zhou, X., Kochian, L. V., & Li, L. (2007). Biochemical and molecular characterization of the homocysteine S-methyltransferase from broccoli (*Brassica oleracea* var. *italica*). *Phytochemistry*, *68*(8), 1112-1119.

Ma, J., Li, J., Zhao, J., Zhou, H., Ren, F., Wang, L., Gu, C., Liao, L. & Han, Y. (2014). Inactivation of a Gene Encoding Carotenoid Cleavage Dioxygenase (CCD4) Leads to Carotenoid-Based Yellow Coloration of Fruit Flesh and Leaf Midvein in Peach. *Plant Molecular Biology Reporter*, *32*(1), 246-257.

Marin, E., Nussaume, L., Quesada, A., Gonneau, M., Sotta, B., Hugueney, P., Frey, A. & Marion-Poll, A. (1996). Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. *The EMBO Journal*, *15*(10), 2331.

Mathieu, S., Terrier, N., Bigey, F., & Günata, Z. (2005). A carotenoid cleavage dioxygenase from *Vitis vinifera* L.: functional characterization and expression during grape berry development in relation to C13-norisoprenoid accumulation. *Journal of Experimental Botany*, *56*(420), 2721-2731.

Mayne, S. T. (1996). Beta-carotene, carotenoids, and disease prevention in humans. *The FASEB Journal*, *10*(7), 690-701.

Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara, S., Saito, T., Ohtani, T. & Miki, W. (1995). Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. *Journal of Bacteriology*, *177*(22), 6575-6584.

Moraga, Á. R., Rambla, J. L., Ahrazem, O., Granell, A., & Gómez-Gómez, L. (2009). Metabolite and target transcript analyses during Crocus sativus stigma development. *Phytochemistry*, *70*(8), 1009-1016.

Müller, P., Li, X. P., & Niyogi, K. K. (2001). Non-photochemical quenching. A response to excess light energy. *Plant Physiology*, *125*(4), 1558-1566.

Naqvi, S., Zhu, C., Farré, G., Ramessara, K., Bassie, L., Breitenbach, J., Perez Conesa, D., Ros, G., Sandmann, G., Capell, T. & Christou, P. (2009). Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. *Proceedings of National Academy of Sciences, 106*, 7762–7767.

Niyogi, K. K. (1999). Photoprotection revisited: genetic and molecular approaches. *Annual review of plant biology*, *50*(1), 333-359.

Ohmiya, A. (2009). Carotenoid cleavage dioxygenases and their apocarotenoid products in plants. *Plant Biotechnology*, *26*(4), 351-358.

Ohmiya, A., Kishimoto, S., Aida, R., Yoshioka, S., & Sumitomo, K. (2006). Carotenoid cleavage dioxygenase (CmCCD4a) contributes to white color formation in chrysanthemum petals. *Plant Physiology*, *142*(3), 1193-1201.

Ohmiya, A., Sumitomo, K., & Aida, R. (2009). "Yellow Jimba": suppression of carotenoid cleavage dioxygenase (CmCCD4a) expression turns white chrysanthemum petals yellow. *Journal of the Japanese Society for Horticultural Science*, *78*(4), 450-455.

Paine, J. A., Shipton, C. A., Chaggar, S., Howells, R. M., Kennedy, M. J., Vernon, G., Wright, S., Hinchliffe, E., Adams, J., Silverstone, A. & Drake, R. (2005). Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nature biotechnology*, *23*(4), 482-487.

Palaisa, K.A., Morgante, M., Williams, M., Rafalski, A. (2003). Contrasting effects of selection on sequence diversity and linkage disequilibrium at two phytoene synthase loci. *Plant Cell*, *15*, 1795–1806.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic acids research*, *29*(9), e45-e45.

Pfündel, E., & Bilger, W. (1994). Regulation and possible function of the violaxanthin cycle. *Photosynthesis Research*, *42*(2), 89-109.

Qin, X., & Zeevaart, J. A. D. (1999). The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proceedings of the National Academy of sciences*, *96*(26), 15354-15361.

Quinlan, R.F., Jaradat, T.T. and Wurtzel, E.T. (2007). *Escherichia coli* as a platform for functional expression of plant P450 carotene hydroxylases. ***Archives of biochemistry and biophysics***, *458*, 146–157.

Rodrigo, M. J., Alquézar, B., Alós, E., Medina, V., Carmona, L., Bruno, M., Al-Babili, S. & Zacarías, L. (2013). A novel carotenoid cleavage activity involved in the biosynthesis of Citrus fruit-specific apocarotenoid pigments. *Journal of experimental botany*, ert260.

Rodríguez-Concepción, M. (2006). Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells. *Phytochemistry Reviews*, *5*(1), 1-15.

Rodríguez -Concepción, M., & Boronat, A. (2002). Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. *Plant physiology*, *130*(3), 1079-1089.

Rodríguez-Concepción, M., Forés, O., Martínez-García, J. F., González, V., Phillips, M. A., Ferrer, A., & Boronat, A. (2004). Distinct light-mediated pathways regulate the biosynthesis and exchange of isoprenoid precursors during Arabidopsis seedling development. *The Plant Cell*, *16*(1), 144-156.

Römer, S., Fraser, P. D., Kiano, J. W., Shipton, C. A., Misawa, N., Schuch, W., & Bramley, P. M. (2000). Elevation of the provitamin A content of transgenic tomato plants. *Nature biotechnology*, *18*(6), 666-669.

Rosati, C., Aquilani, R., Dharmapuri, S., Pallara, P., Marusic, C., Tavazza, R., Bouvier, F., Camara, B. & Giuliano, G. (2000). Metabolic engineering of beta-carotene and lycopene content in tomato fruit. *The Plant Journal*, *24*(3), 413-420.

Rubio, A., Rambla, J. L., Santaella, M., Gómez, M. D., Orzaez, D., Granell, A., & Gómez-Gómez, L. (2008). Cytosolic and plastoglobule-targeted carotenoid dioxygenases from Crocus sativus are both involved in β-ionone release. *Journal of Biological Chemistry*, *283*(36), 24816-24825.

Rubio-Moraga, A., Ahrazem, O., Pérez-Clemente, R. M., Gómez-Cadenas, A., Yoneyama, K., López-Ráez, J. A., Molina, R.V. & Gómez-Gómez, L. (2014b). Apical dominance in saffron and the involvement of the branching enzymes CCD7 and CCD8 in the control of bud sprouting. *BMC plant biology*, *14*(1), 171.

Rubio-Moraga, A., Rambla, J. L., Fernández-de-Carmen, A., Trapero-Mozos, A., Ahrazem, O., Orzáez, D., Granell, A. & Gómez-Gómez, L. (2014a). New target carotenoids for CCD4 enzymes are revealed with the characterization of a novel stress-induced carotenoid cleavage dioxygenase gene from Crocus sativus. *Plant molecular biology*, *86*(4-5), 555-569.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning* (Vol. 2, pp. 14-9). New York: Cold spring harbor laboratory press.

Schwartz, S. H., Qin, X., & Loewen, M. C. (2004). The biochemical characterization of two carotenoid cleavage enzymes from Arabidopsis indicates that a carotenoid-derived compound inhibits lateral branching. *Journal of Biological Chemistry*, *279*(45), 46940-46945.

Schwartz, S. H., Tan, B. C., Gage, D. A., Zeevaart, J. A., & McCarty, D. R. (1997). Specific oxidative cleavage of carotenoids by VP14 of maize. *Science*, *276*(5320), 1872-1874.

Schwartz, S. H., Tan, B. C., McCarty, D. R., Welch, W., & Zeevaart, J. A. D (2003). Substrate specificity and kinetics for VP14, a carotenoid cleavage dioxygenase in the ABA biosynthetic pathway. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1619*(1), 9-14.

Schwartz, S.H., Qin, X.Q., Zeevaart, J.A.D. (2001) Characterization of a novel carotenoid cleavage dioxygenase from plants. *Journal of Biological Chemistry*, 276:25208–25211

Seo, M., & Koshiba, T. (2002). Complex regulation of ABA biosynthesis in plants. *Trends in plant science*, *7*(1), 41-48.

Simkin, A. J., Moreau, H., Kuntz, M., Pagny, G., Lin, C., Tanksley, S., & McCarthy, J. (2008). An investigation of carotenoid biosynthesis in *Coffea canephora* and *Coffea arabica*. *Journal of plant physiology*, *165*(10), 1087-1106.

Simkin, A. J., Schwartz, S. H., Auldridge, M., Taylor, M. G., & Klee, H. J. (2004b). The tomato carotenoid cleavage dioxygenase 1 genes contribute to the formation of the flavor volatiles β-ionone, pseudoionone, and geranylacetone.*The Plant Journal*, *40*(6), 882-892.

Simkin, A. J., Underwood, B. A., Auldridge, M., Loucas, H. M., Shibuya, K., Schmelz, E., Clark, D.G. & Klee, H. J. (2004a). Circadian regulation of the PhCCD1 carotenoid cleavage dioxygenase controls emission of β-ionone, a fragrance volatile of petunia flowers. *Plant physiology*, *136*(3), 3504-3514.

Tan, B. C., Cline, K., & McCarty, D. R. (2001). Localization and targeting of the VP14 epoxy-carotenoid dioxygenase to chloroplast membranes. *The Plant Journal*, *27*(5), 373-382.

Tan, B. C., Joseph, L. M., Deng, W. T., Liu, L., Li, Q. B., Cline, K., & McCarty, D. R. (2003). Molecular characterization of the Arabidopsis 9-cis epoxycarotenoid dioxygenase gene family. *The Plant Journal*, *35*(1), 44-56.

Tan, B. C., Schwartz, S. H., Zeevaart, J. A., & McCarty, D. R. (1997). Genetic control of abscisic acid biosynthesis in maize. *Proceedings of the National Academy of Sciences*, *94*(22), 12235-12240.

Taylor, M., & Ramsay, G. (2005). Carotenoid biosynthesis in plant storage organs: recent advances and prospects for improving plant food quality. *Physiologia Plantarum*, *124*(2), 143-151.

Tian, L., Magallanes-Lundback, M., Musetti, V., DellaPenna, D. (2003). Functional analysis of beta- and epsilon-ring carotenoid hydroxylases in Arabidopsis. Plant Cell, 5, 1320–1332.

Tuan, P. A., & Park, S. U. (2013). Molecular cloning and characterization of cDNAs encoding carotenoid cleavage dioxygenase in bitter melon (*Momordica charantia*). *Journal of plant physiology*, *170*(1), 115-120.

Tuan, P.A., Kim, J.K., Lee, S., Chae, S.C. & Park, S.U. (2013) Molecular Characterization of Carotenoid Cleavage Dioxygenases and the Effect of Gibberellin, Abscisic Acid, and Sodium Chloride on the Expression of Genes Involved in the Carotenoid Biosynthetic Pathway and Carotenoid Accumulation in the Callus of *Scutellaria baicalensis* Georgi. *Journal of Agricultural & Food Chemistry*, *61***,** 5565-5572.

United Nations Children's Fund (UNICEF). (2010). The number of pre-school age children who suffer from clinical Vitamin A deficiency is 5.2 million. Available from: http:// www. unicef.org/factoftheweek/index_54116.html (Accessed: 20.11.2014).

Vallabhaneni, R., Bradbury, L. M., & Wurtzel, E. T. (2010). The carotenoid dioxygenase gene family in maize, sorghum, and rice. *Archives of biochemistry and biophysics*, *504*(1), 104-111.

Vogel, J. T., Tan, B. C., McCarty, D. R., & Klee, H. J. (2008). The carotenoid cleavage dioxygenase 1 enzyme has broad substrate specificity, cleaving multiple carotenoids at two different bond positions. *Journal of Biological Chemistry*, *283*(17), 11364-11373.

Von Lintig, J. (2010). Colors with functions: elucidating the biochemical and molecular basis of carotenoid metabolism. *Annual review of nutrition*, *30*, 35-56.

Welsch, R., Arango, J., Bär, C., Salazar, B., Al-Babili, S., Beltrán, J., Chavarriaga, P., Ceballos, H., Tohme, J. & Beyer, P. (2010). Provitamin A accumulation in cassava (*Manihot esculenta*) roots driven by a single nucleotide polymorphism in a phytoene synthase gene. *The Plant Cell Online*, *22*(10), 3348-3356.

Wong, J. C., Lambert, R. J., Wurtzel, E. T., & Rocheford, T. R. (2004). QTL and candidate genes phytoene synthase and ζ-carotene desaturase associated with the accumulation of carotenoids in maize. *Theoretical and Applied Genetics*, *108*(2), 349-359.

Yamamizo, C., Kishimoto, S. and Ohmiya, A. (2010). Carotenoid composition and carotenogenic gene expression during Ipomoea petal development. *Journal of Experimental Botany*, *61*(3), 709-719.

Ye, X., Al-Babili, S., Klo¨ti, A., Zhang, J., Lucca, P., Beyer, P. & Potrykus, I. (2000). Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science*, *287*, 303-305.

Yoshioka, S., Aida, R., Yamamizo, C., Shibata, M., & Ohmiya, A. (2012). The carotenoid cleavage dioxygenase 4 (*CmCCD4a*) gene family encodes a key regulator of petal color mutation in chrysanthemum. *Euphytica*, *184*(3), 377-387.

Zhou, X., Yuan, Y., Yang, Y., Rutzke, M., Thannhauser, T.W., Kochian, L.V. & Li, L. (2009). Involvement of a broccoli COQ5 methyltransferase in the production of volatile selenium compounds. *Plant Physiology*, *151*, 528-540.