# Regulation of the carotenoid biosynthetic pathway in petals of California poppy (*Eschscholzia californica*)

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# Abstract

Carotenoids are essential plant pigments. They function in a wide range of processes including light harvesting in the photosynthetic apparatus, photoprotection against light damage, and pigmentation in flowers and fruits to attract pollinators and seed-dispersal herbivores. Carotenogenesis has been studied extensively in the last century in both photosynthetic and non-photosynthetic tissues of many plant species. Although most of the enzymes and their metabolites of the pathway have been identified, little is still known about how carotenoid production is regulated.

Previous studies have proposed that regulation of the carotenoid pathway is through metabolite feedback occurring at both transcriptional and post transcriptional levels. This thesis examines the evidence for carotenogenesis gene transcription being feedback regulated by changes in carotenoid metabolites in petals of California poppy (*Eschscholzia californica*), and if so, by which metabolite(s).

Virus-induced gene silencing (VIGS) was used to silence carotenoid biosynthetic genes in the petals of orange California poppy. High efficacy of silencing was achieved by first infiltrating and then drenching the California poppy seedlings with the *Agrobacterium tumefaciens* strain GV3101 containing the VIGS vectors. The VIGS vectors included portions of carotenoid gene fragments isolated from California poppy. qRT-PCR confirmed that transcript abundance of the targeted carotenogenesis genes *EcaPDS*, *EcaZDS*, *EcaLCYb*, *EcaCHYb* and *EcaZEP* was significantly reduced in the flower petals. Reduced transcript abundance of all genes apart from *EcaLCYb* altered flower colour. HPLC analyses revealed that the colour altered flower petals with knocked-down expression of each targeted gene resulted in a reduction of total carotenoid content and an altered profile of carotenoids. This manifested as an accumulation of higher amounts of intermediates including phytofluene, ζ-carotene, β-carotene and zeaxanthin, some of which are not usually seen in the flowers, and a reduction of the end products such as *retro*-carotene-triol and eschscholtzxanthin. However, these alterations in carotenoid profiles were not associated with any dramatic changes in transcript abundance of the non-TRV-targeted endogenous genes in the pathway. Therefore, little evidence was found for metabolite feedback regulation of transcriptional activity in the carotenoid biosynthetic pathway from this study. Other possible mechanisms for controlling carotenogenesis are discussed.

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To my parents, family and friends, thanks for your support and understanding, so I can concentrate on finishing this thesis. To my heavenly father, I know you are there for me all the time conferring and strengthen, I am nothing without you. Unless the LORD builds the house, its builders labour in vain. Unless the LORD watches over the city, the watchmen stand guard in vain. Psalm 127:1

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# List of abbreviations

| | |
|---|---|
| h: | hours |
| sec: | seconds |
| min: | minutes |
| dpi: | days post inoculation |
| ˚C: | degrees Celsius |
| U: | enzyme unit |
| V: | voltage |
| g: | gram |
| *g*: | *g*-force or gravity |
| rpm: | revolution per minute |
| mL: | millilitre |
| M: | molar; moles per litre |
| mM: | millimolar; millimole per litre |
| μM: | micromolar; micromole per litre |
| mg: | milligram |
| ng: | nanogram |
| amu: | atomic mass unit |
| w/v: | weight-to-volume ratio |
| v/v: | volume-to-volume ratio |
| *m/z*: | mass-to-charge ratio |
| DW: | dry-weight |
| bp: | base pair |
| Kb: | kilo base pair |
| DNA: | deoxyribonucleic acid |
| cDNA: | complementary deoxyribonucleic acid |
| RNA: | ribonucleic acid |
| rRNA: | ribosomal RNAs |
| siRNA: | small interfering RNA |
| miRNA: | microRNA |
| dNTP: | deoxy-nucleotide- triphosphate |

| | |
|---|---|
| TM: | melting temperature |
| LB: | Luria-Bertani broth |
| DMSO: | dimethyl sulfoxide |
| EDTA: | ethylenediaminetetraacetic acid |
| SDS: | sodium dodecyl sulfate |
| MES: | 2-(N-morpholino)ethanesulfonic acid |
| TBE: | Tris/Borate/EDTA |
| BHT: | butylated hydroxytoluene |
| PVP: | polyvinylpyrrolidone |
| MOPS: | 3-(N-morpholino)propanesulfonic acid |
| CPTA | 2-(4-chlorophenylthio)triethylamine hydrochloride |
| HPLC: | high performance liquid chromatography |
| LC-MS: | liquid chromatography–mass spectrometry |
| GMO: | genetically modified organism |
| PC2: | physical containment level 2 |
| UV: | ultra violet |
| OD: | optical density |
| milliQ: | purified water using Milli-Q ultrapure system |
| CaMV 35S: | 35S promoter, from cauliflower mosaic virus |
| STDEV: | standard deviation |

# CHAPTER ONE
# INTRODUCTION

## 1.1 General introduction to carotenoids

### 1.1.1 Discovery of carotenoids

Carotenoids are one of four naturally occurring pigments (carotenoids, anthocyanins, betalains and chlorophylls) that are responsible for the colour we see in the plant world. Carotenoids are yellow, orange and red in colour. The mixing of the carotenoid-based colours caused by their differential accumulation together with the presence of other pigments lead to a wide variety of hues. The first carotenoid isolated was β-carotene. It was crystallised by Wachenroder in 1831 from a carrot extract and named carotene. A second yellow carotenoid was isolated shortly after by Berzelius in 1837 from an autumn leaf extract and named xanthophyll (from Greek xanthos [yellow] and phyllon [leaf]). In 1907, Richard Willstatter determined the empirical formula of carotenoids as $C_{40}$ (Figure 1.1). Tswett then in 1911, using chromatography, isolated additional pigments that had the same empirical formula and which together with β-carotene and xanthophylls were collectively named carotenoids (cited in Eugster 1995). Today there are more than 600 known carotenoids (Rodriguez-Amaya 2001), which have been grouped into two classes: carotenes (hydrocarbons that do not contain oxygen) and xanthophylls (which contain oxygen) (Figure 1.1).

### 1.1.2 Chemical structure of carotenoids

Chemically carotenoids are $C_{40}$ tetraterpenoid molecules. They consist of eight isoprene units arranged head to tail except at the middle, which gives them a symmetrical structure (Figure 1.1) (Namitha & Negi 2010). The basic linear hydrocarbon structure of the carotenoid can undergo cyclisation at either or both ends and have hydroxyl and oxygen groups added. The number and the position of the double bonds, the isomeric forms, addition of the hydroxyl groups and oxygen molecules give rise to different structures that result in the varied colours and different antioxidant properties of the carotenoids.

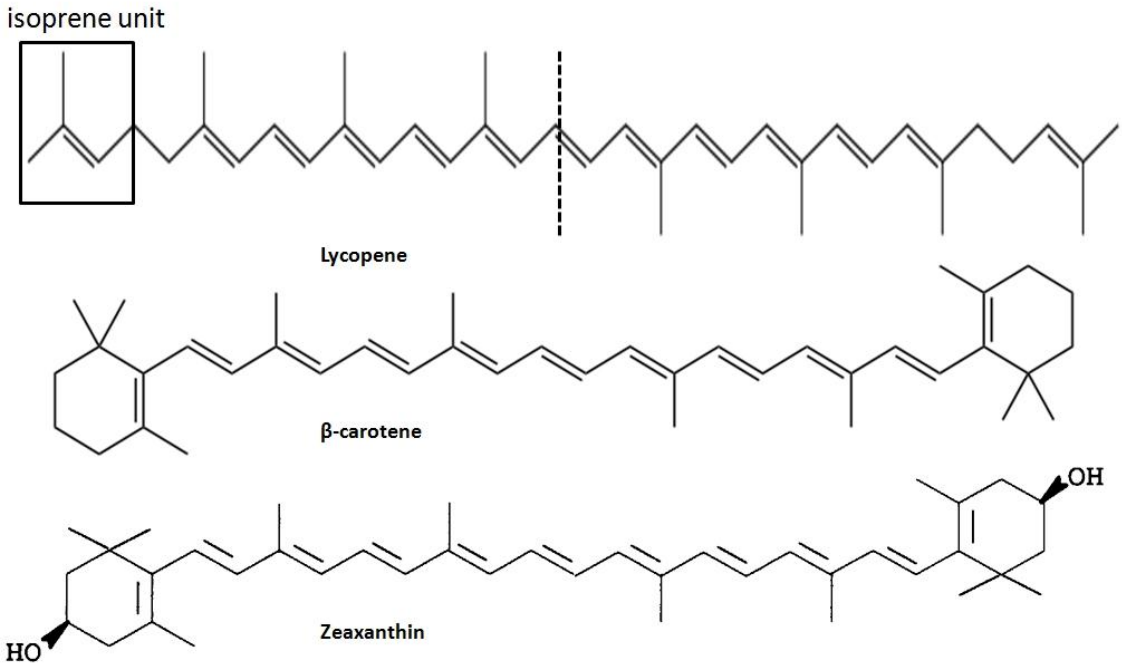



**Figure 1.1:** Representative structures of carotenoids. Carotenes, which do not contain oxygen are represented by lycopene (linear) and β-carotene (cyclised) molecules. Xanthophylls, which do contain oxygen are represented by zeaxanthin. The dashed line crossed at the centre of the lycopene chemical structure represents the symmetrical point of the representative carotenoid. (Namitha & Negi 2010)

### 1.1.3 Biological function of carotenoids

Carotenoid biosynthesis occurs in all photosynthetic organisms from cyanobacteria, green algae through to all higher plants as well as in some non photosynthetic bacteria and fungi (Simpson et al. 1964; Takaichi & Mochimaru 2007; Cazzonelli 2011). In eukaryotes, carotenoids are produced and stored in the chloroplasts and chromoplasts. The carotenoid colour of the chloroplast is normally masked by chlorophyll and usually becomes prominent in autumn only when the chlorophyll component is degraded. Chloroplast-located carotenoids have essential functions in photosynthesis. For example, zeaxanthin helps in light harvesting, stabilising the thylakoid membrane and protecting the photosynthetic reaction centre from photo-oxidation by dispersing the excess light energy obtained from the antenna pigments (Cunningham & Gantt 1998; Baroli & Niyogi 2000; Davison et al. 2002; Niinemets et al. 2003). Their role in light harvesting is to broaden the effective light spectrum for photosynthesis (Marin et al. 2011). The carotenoid composition of chloroplasts varies little presumably because of the important role of carotenoids in photosynthesis. By contrast, the carotenoid composition in chromoplasts of flowers and fruits varies greatly presumably because it provides advantages for attracting pollinators and seed-dispersal-herbivores to the flowers and fruits.

Some carotenoids function as precursors of plant growth hormones such as abscisic acid (ABA) and strigolactones (Jiang et al. 2008). Others provide the colours for fruits, flowers, some fungi and bacteria which may also function to protect these organisms from light and oxidative damage (Hertzberg et al. 1976). The scent of some flowers is also produced when β-carotene and α-carotene are cleaved by carotenoid cleavage dioxygenases (CCDs) (Baldermann et al. 2010). With the exception of aphids, animals do not appear to be able to biosynthesize carotenoids, but some do accumulate carotenoids in different parts of their bodies by ingesting them (Moran & Jarvik 2010). For example, carotenoids are responsible for the pink flesh of salmon, the yellow colour of egg yolks and the red feathers of flamingos.

Carotenoids are an important part of the human diet as they are a source of vitamin A, light protection of the eyes and antioxidants. In the early 1930s, biochemists discovered that the carotenoids that contain a β-ring such as β-carotene are metabolised to vitamin A in rats. These carotenoids were hence termed provitamin A carotenoids. Intake of provitamin A type carotenoids is important for preventing night blindness (Pirie 1983). Increased dietary intake

of zeaxanthin and lutein has been linked to reduced risk of age-related macular degeneration (Delcourt et al. 2006). In addition to their well established role in protecting sight, carotenoids are antioxidants that help scavenge free radicals that can cause DNA damage and affect the function of immune system (Azqueta & Collins 2012; Rossoni et al. 2012). Carotenoids such as lycopene have reported anticancer activity (Levy et al. 1998), which has been attributed to inhibiting unregulated cell proliferation by promoting better cell to cell communication (Bhuvaneswari & Nagini 2005).

### 1.1.4 Importance of carotenoid in industry

Carotenoids have attracted research attention from the ornamental and fruit industries. Carotenoids are of interest to the cut flower industry because of the variation in colour which can be achieved by engineering of its biosynthetic pathway (Zhu et al. 2010). Similarly, breeders of carotenoid-coloured fruits such as watermelon, citrus and tomato are interested in manipulating carotenoid content for both aesthetic and health reasons. A deeper understanding of the regulatory mechanisms of carotenoid production in plants will provide great insight for fruit and ornamental plant breeders working on augmenting carotenoid content.

Carotenoids are also used as natural dyes, additives and most recently as sensitizers in dye-sensitised solar cells (Narayan 2012; Wrolstad & Culver 2012). They can be harvested from natural sources or be chemically synthesised. Carotenoids are used in food and cosmetic products. Depending on the sources, β-carotene and lutein are used either as food colouring or as an additive in animal feed. β-carotene is used to provide colour for oil, margarine and fruit juice; lutein is used in chicken feed for the colour of egg yolk and skin (Rymbai et al. 2011). Astaxanthin is fed to salmon to enhance flesh colour (Wrolstad & Culver 2012). Recently, researchers have successfully used naturally occurring pigments such as carotenoids to replace the ruthenium complex as the photosensitiser in regenerative solar cells. The dye-sensitizer solar cell has the potential to become the green energy of tomorrow (Yamazaki et al. 2007).

## 1.2 Biosynthesis and storage of carotenoids

### 1.2.1 Biosynthesis of the carotenoid precursors

The 20-carbon precursor of carotenoids, geranylgeranyl diphosphate (GGPP) is formed by the condensation of 3 molecules of 5-carbon isopentenyl diphosphate (IPP) with 1 molecule of its isomer dimethylallyl diphosphate (DMAPP). In plants, IPP and DMAPP can be made *via* two independent pathways; the plastid localised methylerythritol 4-phosphate (MEP) pathway and the cytosol-specific mevalonic acid (MVA) pathway. In animals and fungi, these substrates are made only by the MVA pathway as the MEP pathway is not present. In plants, carotenoids were initially thought to be made only from the IPP and DMAPP synthesised *via* the plastid localised MEP pathway because movement of DMAPP and IPP from cytosol to plastid was found to be limited (Rohmer 1999; Rodriguez-Concepcion & Boronat 2002). More recent research has shown that some cytosolic produced DMAPP and IPP does move into the plastid in *Arabidopsis* (Flores-Perez et al. 2010), although the amounts appear to be small because blocking the MVA pathway does not affect carotenoid accumulation (Rohmer 1999). Hence it is currently accepted that carotenoids appear to be synthesised predominantly from the isoprenoid precursors produced *via* the plastid localised MEP pathway (Rodriguez-Concepcion 2010).

Biosynthesis of the carotenoid precursors IPP and DMAPP begins with 1-deoxy-D-xylulose-5-phosphate synthase (DXS) catalysing the condensation of the glycolytic metabolites pyruvate and glyceraldehyde-3-phosphate (GAP) into 1-deoxy-D-xylulose-5-phosphate (DXP) (Figure 1.2). DXP is then converted by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) to 2-C-methyl-D-erythritol 4-phosphate (MEP) (Bouvier et al. 1998). MEP is then sequentially converted by CDP-ME kinase (CMK), ME-cPP synthase (MDS), HMBPP synthase (HDS) and HMBPP reductase (HDR) to IPP and its inter-convertible isomer DMAPP (Meyer et al. 2003; Ahn & Pai 2008). The MEP pathway produces both IPP and DMAPP by the reaction of HDR. The isomerisation between IPP and DMAPP can be catalysed by IPP/DMAPP isomerase (IDI) when they are produced in cytosol and mitochondria *via* MVA pathway (Zhang et al. 2007). The plastid localised IPP and DMAPP are converted to geranylgeranyl diphosphate (GGPP) by GGPP synthase (GGPS) (Thabet et

al. 2012). GGPP is the precursor for a number of compounds such as carotenoids, gibberellins and the chlorophyll side chain (Walter & Strack 2011) (Figure 1.2).

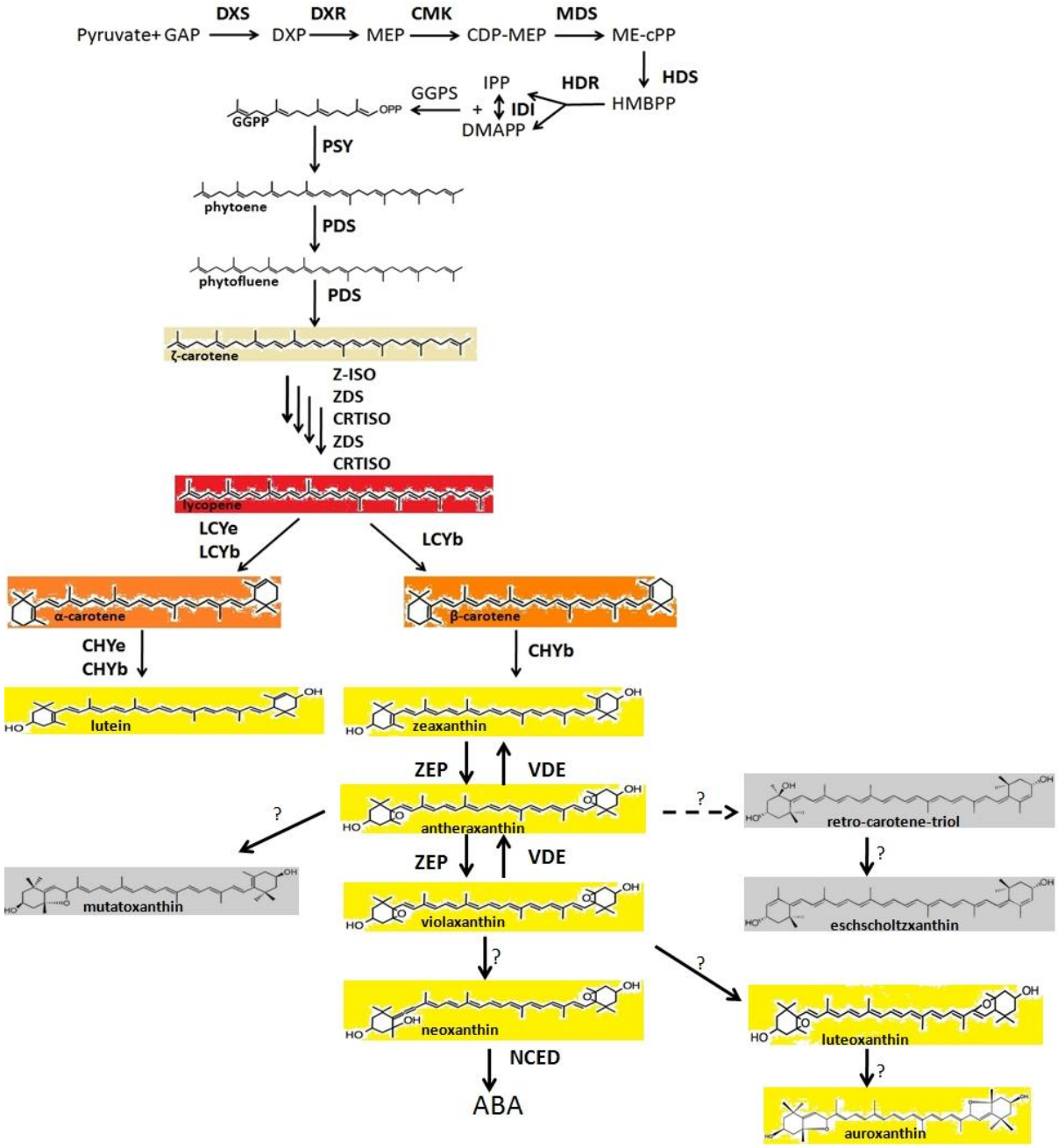



**Figure 1.2:** The carotenoid biosynthetic pathway, its precursors and its connection to ABA synthesis. Figure modified from Liotenberg 1999, Zhu 2010 and Maoka 2000. The question marks (?) and dashed line represent the unknown enzymes and the proposed pathways for the shown metabolites

IPP (isopentenyl diphosphate), DMAPP (dimethylallyl diphosphate), GAP (glyceraldehydes 3-phosphate ), DXP (1-deoxyxylulose-5-phosphate ), MEP (2-c-methyl-D-erythritol), CDP-ME (CDP-ME 2-phosphate), ME-Cpp (2-C-methyl-D-erythritol 2,4-cyclodiphosphate), HMBPP (1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate) and GGPP (geranylgeranyl diphosphate)

### 1.2.2 Biosynthetic pathway of carotenoids

#### *1.2.2.1 Identification of the enzymes and genes of the carotenoid biosynthetic pathways*

Enzymes and their corresponding metabolites in the carotenoid biosynthetic pathway were identified in plants by studying naturally occurring mutations, using chemical inhibitors of the pathway and by growing plants under specific abiotic conditions (Spurgeon & Porter 1980). Some carotenoid genes, e.g., phytoene desaturase (*PDS*) and β-lycopene cyclase (*LCYb*) were first isolated from cyanobacteria (Chamovitz et al. 1991; Cunningham et al. 1993) and subsequently used to probe plant cDNA libraries to identify their corresponding plant orthologs. The ripening related sequence pTOM5 of tomato was eventually found to be phytoene synthase by studying pTOM5 silenced plants and finding pale coloured flowers and yellow ripe fruits (Bird et al. 1991). Zeaxanthin epoxidase (ZEP) was identified in tobacco plants by transposon tagging (Marin et al. 1996). After discovery, over-expressing the plant carotenogenesis genes in *E.coli* cells has been a commonly used method to confirm the function of the isolated plant gene products (Lotan & Hirschberg 1995).

#### *1.2.2.2 Reactions of the plant carotenoid biosynthetic pathway*

The basic pathway for carotenoid biosynthesis has been known for many years and it is outlined in Figure 1.2 (Simpson et al. 1964). Condensation of two GGPP molecules into phytoene by phytoene synthase (PSY) is the first committed step in the pathway (Figure 1.2). Phytoene is then dehydrogenated to ζ-carotene by phytoene desaturase (PDS) and to lycopene by ζ-carotene desaturase (ZDS). Cyclisation of lycopene is an important branch point of the carotenoid biosynthetic pathway in which lycopene is converted either to α-carotene or β-carotene. Conversion to α-carotene is catalysed by the combined action of β-lycopene cyclase (LCYb) and ε-lycopene cyclase (LCYe), whereas conversion to β-carotene is catalysed just by β-lycopene cyclase (LCYb). α-carotene is converted to lutein by the sequential action of β-carotene hydroxylase (CHYb) and ε-carotene hydroxylase (CHYe). β-carotene is converted to zeaxanthin by β-carotene hydroxylase (CHYb). Zeaxanthin is converted to violaxanthin by zeaxanthin epoxidase (ZEP) (Zhu et al. 2010). The violaxanthin can then be further converted to ABA (Bang et al. 2006). In recent years, more detail has emerged on the individual steps of the carotenoid biosynthetic pathway, which will be discussed in more detail in the following paragraphs.

For instance, the dehydrogenation of phytoene to phytofluene and then to ζ-carotene, which is catalysed by PDS requires the help of a plastid-targeted alternative oxidase (PTOX) (Josse et al. 2000). The conversion of ζ-carotene to lycopene by ZDS is a multistep process requiring the action of two isomerases, ζ-carotene isomerase (Z-ISO) and carotene isomerase (CRTISO). They convert ζ-carotene into the isomeric form favoured by ZDS, as ζ-carotene can exist in various *cis* and *trans* isomers because of its many double bonds. To do this ζ-carotene is first isomerised by Z-ISO to enable ZDS to convert it to neurosporene (Chen et al. 2010b). This isomerisation may also be partially complemented by light-mediated photoisomerisation (Li et al. 2007). CRTISO then isomerises neurosporene to an intermediate, which is then converted by ZDS to prolycopene (Isaacson et al. 2002; Isaacson et al. 2004). Prolycopene is then finally isomerised by CRTISO to produce all-*trans*-lycopene (Isaacson et al. 2002; Isaacson et al. 2004). Intriguingly, this complex set of reactions in plants that converts phytoene to ζ-carotene to all-*trans*-lycopene is catalysed in bacteria by a single enzyme CrtI (Linden et al. 1991).

Lycopene cyclisation splits the carotenoid biosynthetic pathway into two branches dependent on the relative activities of LCYb and LCYe. LCYb catalyses the formation of the β-ring, whereas LCYe catalyses the formation of the ε-ring. The formation of a β-ring on one end and ε-ring on the other produces α-carotene, whereas formation of β-rings on both ends produces β-carotene (Cunningham et al. 1996). Hydroxyl groups are then added to the ring structures by the action of CHYe (for the ε-ring) and CHYb (for the β-ring). β-carotene is converted to β-cryptoxanthin by the addition of a hydroxyl group to one of its β-rings and is then converted to zeaxanthin by the addition of another hydroxyl group to its second β-ring. Similarly, α-carotene is converted to either α-cryptoxanthin or zeinoxanthin depending on whether the ε-ring or β-ring is hydroxylated first. Subsequent addition of one more hydroxyl groups to the remaining ring of α-cryptoxanthin/zeinoxanthin results in formation of lutein (Kim et al. 2009).

Following hydroxylation carotenoids undergo epoxidation, which adds oxygen molecules to the ring structure. ZEP converts zeaxanthin to antheraxanthin by the addition of a single oxygen molecule to one of its β-rings. The same enzyme then converts antheraxanthin to violaxanthin by the addition of another oxygen molecule to its second β-ring (Thompson et al. 2000). This process can be reversed by violaxanthin de-epoxydase (VDE) in the xanthophyll cycle, which is essential for photoprotection of the photosynthetic apparatus. Under light

stress, violaxanthin is de-epoxidised to antheraxanthin and zeaxanthin to help dissipate excess light energy as heat. Following alleviation of the light stress, the de-epoxidation can be reversed by ZEP (DemmigAdams & Adams 1996). Intriguingly, the xanthophyll cycle might also operate under drought stress to produce zeaxanthin for reducing lipid oxidation (Fini et al. 2012). Antheraxanthin can undergo epoxide furanoside rearrangements to mutatoxanthin and neoxanthin, while violaxanthin can undergo an epoxide furanoside rearrangement to auroxanthin *via* luteoxanthin (Rodriguez-Amaya 2001).

All-*trans*-violaxanthin and all-*trans*-neoxanthin can be isomerised to their 9-*cis* isomers, which when cleaved by 9-*cis*-epoxy-carotenoid dioxygenase (NCED) yields ABA. In some marine bacteria and plants, additional reactions of the carotenoid pathway produce ketocarotenoids (e.g. astaxanthin) and retro-carotenoids (e.g. eschscholtzxanthin). The ketocarotenoid biosynthetic pathway is better understood. The Haematococcus oxygenase (crtO) 3,3-β-hydroxylase (crtZ) and 4,4-β-oxygenase (crtW) enzymes all have a role in introducing keto and hydroxyl groups into carotenoids to produce ketocarotenoids (Ralley et al. 2004). By contrast, less is known about the formation of retro-carotenoids such as eschscholtzxanthin (Strain 1937; Williams et al. 1966; Maoka et al. 2000). It has been proposed that eschscholtzxanthin is derived from antheraxanthin *via* a *retro*-carotene-triol (Andrewes et al. 1979; Maoka et al. 2000).

### 1.2.3 Storage of carotenoids in plastids

Carotenoids are synthesised and stored in both chloroplasts and chromoplasts, but only aspects related to chromoplasts will be emphasised here. There are five types of chromoplast ultrastructure that can be distinguished: globular, membranous, tubular, reticulo-globular and crystalline (Ljubesic et al. 1991). The globular chromoplast is the major type found in mango (Vasquez-Caicedo et al. 2006), whereas the tubular chromoplast is the most abundant type in banana and saffron (Caiola & Canini 2004). Tomato fruits accumulate lycopene in membrane-shaped crystalloid plastids (Rosso 1968). The carotenoid composition and type of lipid and protein present are thought to be the key factors controlling which type of chromoplast develops. Chromoplasts differentiate directly from proplastids or by differentiation from amyloplasts and chloroplasts (Marano et al. 1993). Chromoplasts most commonly develop from chloroplasts, which are modified at the ultrastructural level to accommodate the new function in carotenoid sequestration rather than its prior role in photosynthesis. The

appropriate carotenoid sequestration system prevents the deleterious effects that happen from having excess cellular carotenoid content (Egea et al. 2010).

Many carotenoids are modified after their biosynthesis by the attachment of different ester groups. Esterification and other modifications of carotenoids are thought to stabilise them and so aid storage (Atienza et al. 2007). Esterification of lutein is thought to protect it from UV and heat damage (Subagio et al. 1999). Different ester groups can be added to the same carotenoid molecule which results in multiple peaks in their HPLC profile. The components of carotenoid esters can be resolved by saponification, a process that uses alkali to remove the attached ester groups (Yuan & Chen 1999).

## 1.3 Regulation of carotenoid biosynthesis

Because of its necessity for life, the carotenoid biosynthetic pathway is tightly regulated. Although the enzymatic steps in the carotenoid biosynthetic pathway are well established, relatively little is still known about how these steps are regulated (Cunningham & Gantt 1998). Experimental data suggests the pathway is regulated by signals related to the biological roles of carotenoids, and so environmental factors, developmental cues, accumulation of metabolites and storage conditions are signals that regulate the carotenoid biosynthetic pathway. The pathway is also controlled by transcriptional and post-transcriptional regulations (Ruiz-Sola & Rodriguez-Concepcion 2012). In addition, some biosynthetic steps have been found to be more rate limiting than others (Cazzonelli & Pogson 2010).

### 1.3.1 Signals regulating carotenoid biosynthesis

#### *1.3.1.1 Light*

Light induces the carotenoid biosynthetic pathway during plant development, which is consistent with its function in producing carotenoids for both light harvesting and photoprotection. Conversely, transcriptional suppression of the carotenoid pathway occurs in darkness. In *Arabidopsis* seedlings, the suppression was caused by direct interaction of the phytochrome interaction factor 1 (PIF1) with the *PSY* gene promoter to suppress its expression (Toledo-Ortiz et al. 2010). Toledo-Ortiz et al. proposed that the expression of other carotenoid genes may also be regulated by other PIFs but indirectly (Toledo-Ortiz et al. 2010). Light-induced accumulation of carotenoid gene transcripts has also been observed in

the green alga *Chlamydomonas reinhardtii* upon transfer from dark to light (Sun et al. 2010). Light differentially affects the normal accumulation of carotenoids in carrot roots. For example, the underground portion of the carrot root normally accumulates β- and α-carotene. However, when exposed to light it accumulates lutein, like the carrot leaves. The total carotenoid content of the underground part of the root was also higher than in the light exposed part (Fuentes et al. 2012). Light catalysed non-enzymatic isomerisation may also occur in the carotenoid biosynthetic pathway because Z-ISO defective mutants are still able to produce some carotenoids in light-exposed tissue (Chen et al. 2010b).

*1.3.1.2 Circadian clock*

The circadian clock has been shown to influence carotenoid biosynthesis or metabolism. In *Arabidopsis*, expression of many carotenoid biosynthetic genes fluctuate with the day/night cycle and some of these genes continue this rhythmic fluctuation when the plant is moved to either constant light or dark (Sun et al. 2010). A transcript encoding a carotenoid cleavage dioxygenase (*OfCCD1*) identified from flowers of *Osmanthus fragrans* was found to show a photorhythmic increase in abundance in dark (Baldermann et al. 2010). This was proposed to be a mechanism for optimising fragrance emission by the flowers at night. In leaves of *Nicotiana plumbaginifolia*, the transcript abundance of *ZEP* and *NCED* was found to fluctuate with the day/night cycle (Audran et al. 1998; Thompson et al. 2000). An extensive transcriptomic study of the aerial parts of 28-day-old tomato seedling has revealed that transcript abundance of many carotenoid biosynthetic genes follow a diurnal pattern (Facella et al. 2008).

*1.3.1.3 Environmental stress*

Environmental stresses can also regulate the biosynthesis of carotenoids, which is consistent with the role of carotenoids in scavenging reactive oxygen species (ROS), preventing oxidative damage of membranes and being precursors of plant hormones that regulate plant stress responses (Apel & Hirt 2004). Carotenoids are produced to alleviate light damage, which is one of the major environmental stresses. Sun-shade acclimation is associated with changes in both carotenoid content and composition. In sun-exposed leaves the total carotenoid content, in particular the carotenoids of the xanthophyll cycle, is greater than in shade-held leaves (Brugnoli et al. 1998). When the carotenoids of the xanthophylls cycle were increased by over-expression of the *CHYb* gene in *Arabidopsis*, these plants showed greater tolerance to environmental stresses such as high light and high temperature and also displayed

less leaf necrosis and anthocyanin production which act as stress indicators (Davison et al. 2002). A volatile stress signal can be induced by light in *Arabidopsis* by oxidation of β-carotene (Ramel et al. 2012). Carotenoids may also help in relieving plants from the effects of drought stress. Under drought conditions the *ZEP* transcript abundance was found to be increased in tomato roots (Thompson et al. 2000). A microarray study has supported the study by Thompson et al. by observing positive correlation between osmotic stress and carotenoid up-regulation in *Arabidopsis* (Meier et al. 2011). Not only drought, but salinity and ABA application also increase the transcript abundance of *PSY3* in *Poaceae* (Li et al. 2008; Welsch et al. 2008). Although salinity did not directly affect carotenoid content, treatment of plants with GA did reduce carotenoid accumulation. It was speculated the perception of saline stress through GA may play a role in regulation of carotenoid accumulation (Maggio et al. 2010).

*1.3.1.4 Developmental signals*

Developmental signals are other important cues for carotenogenesis in flowers and fruits. The total carotenoid content and the composition of Japanese morning glory flowers increases as the green young buds develop into the yellow mature flower (Yamamizo et al. 2010). Carotenoid biosynthesis is also induced during fruit ripening to produce the large amounts of carotenoids that provide colour to appeal to the seed-dispersal-herbivores. In tomatoes, the ripening related APETALA2 transcription factor (SlAP2a) negatively regulates tomato fruit ripening, and transcript silencing of the gene influences flux of metabolites through the carotenoid pathway to cause a colour change in the fruit. (Chung et al. 2010). The relationship between fruit ripening and carotenoid biosynthesis is also indicated by the strong correlation of *PDS* gene expression with chromoplast differentiation (Corona et al. 1996) as the transition of chloroplasts to chromoplasts is an accepted marker of fruit ripening in tomato (Clotault et al. 2008).

*1.3.1.5 Plastid development*

The sequestration of carotenoids in various types of plastids act as sinks for carotenogenesis. Plastid development affects the accumulation of carotenoids due to their sink size, but also regulates carotenogenesis (Cazzonelli & Pogson 2010). It was shown that the specific ultrastructure such as the presence of plastoglobules (PG) in the plastid is related to chromoplast differentiation from chloroplast and correlated directly to synthesis of certain carotenoids (Vanegas-Espinoza et al. 2011). In cauliflower, a change in *Or* gene expression led to a great accumulation of β-carotene in the flower, which is not usually found in the

cauliflower head. Loss function of *Or* results in white coloured cauliflower, whereas over-expression of *Or* induces carotenoid production and accumulation both in cauliflower and in potato tubers (Li et al. 2001; Lopez et al. 2008a). The *Or* gene studies have suggested a tight relationship between chromoplast development and the regulation of carotenogenesis. The increased accumulation of carotenoids is quite often associated with a change of the chromoplast ultrastructure (Galpaz et al. 2008). It was also thought that chloroplast development played a vital role in the regulation of carotenoid accumulation, but direct evidence supporting this is still lacking (Hirschberg 2001).

*1.3.1.6 Carotenoid metabolites*

Over the past decade, evidence has accumulated that the carotenoid biosynthetic pathway is controlled by end product feedback regulation both in photosynthetic and non-photosynthetic tissues. A previous study showed increased *PDS* gene promoter activity in tobacco seedlings due to carotenoids accumulation that was induced by chemical inhibition of the carotenoid biosynthetic pathway. Both Norflurazon and 2-(4-chlorophenylthio)triethylamine hydrochloride (CPTA) were tested on the seedlings, which are the inhibitors of PDS and LCYb respectively (Corona et al. 1996). This work has suggested a potential feedback regulation mechanism of the carotenoid pathway in green tissues. The same work also made possible for researchers to propose that regulatory metabolites affecting transcriptional abundance of carotenoid biosynthetic genes should be formed after the cyclisation step (Corona et al. 1996). An inhibitor study carried out on flowers has again led to similar results, where yellow daffodil flowers dipped in CPTA accumulated lycopene turned orange, and had increased mRNA transcript abundance of *PSY*, *PDS* and *LCY* (Al-Babili et al. 1999). Silencing of the *CHYb* gene in potato tubers increased total carotenoid and β-carotene content and this was accompanied by increased transcript abundance of other carotenoid pathway genes (Diretto et al. 2007b). Over-expression of *PDS* in tomato fruit increased β-carotene content by 3-fold and increased transcript abundance of the endogenous genes *PDS*, *ZDS*, and *LCYb*, but interestingly decreased transcript abundance of *PSY* and did not change the total fruit carotenoid content (Roemer et al. 2000). Over-expression of Erwinia *PDS* (*Crt1*) and *LCY* (*CrtY*) in potato tubers elevated carotenoid content as well as mRNA transcript abundance of the endogenous carotenoid biosynthetic genes (Diretto et al. 2007a). However, other findings indicated that feedback regulation of the transcription of the pathway genes by carotenoid metabolites may not always occur. For example, the elevation of carotenoid content in Golden Rice by introduction of daffodil *PSY* and bacterial carotenoid desaturase

(*CRTI*) did not affect transcript abundance of the endogenous carotenoid genes (Schaub et al. 2005). Therefore, it is still unclear whether the activity of the pathway is controlled by metabolite feedback regulation and is there a specific metabolite responsible for such regulation. But clearly, it is a complicated question in terms of whether feedback regulation occurs at the transcriptional level. (Chen et al. 2010b).

### 1.3.2 Role of carotenoid biosynthetic genes in the regulation of the pathway

#### *1.3.2.1 MEP pathway*

The rate of carotenoid production is limited in part by gene activity of the plastidial MEP isoprenoid pathway (Figure 1.2). *DXS* exists as a small gene family (of 2 to 3 members) in a number of plant species (Walter et al. 2002; Cordoba et al. 2009). The different DXS isoforms are thought to regulate tissue-specific carotenoid accumulation (Cordoba et al. 2009). In *Arabidopsis, DXS* over-expression and down-regulation increased and decreased carotenoid content respectively (Estevez et al. 2001). In tomato fruit, increased *DXS* transcript abundance was positively correlated with *PSY* transcript abundance and increased carotenoid accumulation (Lois et al. 2000). The importance of DXR activity in controlling carotenoid synthesis is less clear. The fact that DXR transcript and protein abundance did not change as carotenoids accumulate in tomato fruit during ripening suggests its constitutive activity is high enough not to limit carotenoid biosynthesis (Rodriguez-Concepcion & Boronat 2002). However, more recently Carretero-Paulet et al (2006) found that 35S:*DXR Arabidopsis* plants that showed increased DXR activity contained higher chlorophyll and carotenoid content than . Another gene of the MEP pathway that is important for carotenoid production is *HDR*. *HDR* transcript abundance significantly increased in tomato fruit as they ripened and in *Arabidopsis* seedlings as they de-etiolated and this correlated with increased production of carotenoids (Botella-Pavia et al. 2004).

#### *1.3.2.2 GGPP to phytoene*

The reaction catalysed by PSY, i.e., the condensation of two GGPP molecules into the 40-carbon phytoene, is considered the first committed and major rate limiting step in the synthesis of carotenoids (Ruiz-Sola and Rodriguez-Conception, 2012). PSY is encoded by a small gene family in many monocot plants such as rice, bread wheat and maize, and by two *PSY* genes in the dicot tomato (Bartley & Scolnik 1993; Gallagher et al. 2004; Li et al. 2008). The different members of the *PSY* small gene family are involved in regulation of

carotenogenesis in different tissue types. For example, both *PSY1* and *2* transcripts were expressed in leaves of rice and maize (white, yellow varieties), but *PSY1* transcripts were only found in the endosperm of the yellow maize variety (Gallagher et al. 2004). The two *PSYs* of tomato have also revealed their specific expression pattern in fruits and green tissues (Bartley & Scolnik 1993). The transcript of *PSY3* is specifically expressed in the root, and is suggested a role to regulate abiotic stress-induced root carotenogenesis (Li et al. 2008). A *PSY* mutation in bread wheat that introduced an alternative splicing site was first recognised as a quantitative trait locus (QTL) for colour determination of wheat flour (Howitt et al. 2009). The variation of the QTL caused by the sequence duplication results in the production of four different PSY transcripts, only two of which are in frame and therefore functional (Howitt et al. 2009). PSY activity is also regulated post-transcriptionally with both active and inactive forms of PSY protein having been identified in white mustard. The active form is associated with the thylakoid membrane, whereas the inactive form is membrane disassociated (Welsch et al. 2000).

*1.3.2.3 Phytoene to lycopene*

Desaturation and isomerisation of uncoloured phytoene to the red all-*trans*-lycopene are also essential reactions for plant viability. In *Arabidopsis*, preventing the four desaturation steps that lead to prolycopene by silencing *PDS* and *ZDS* caused photobleaching phenotypes, retarded growth, and abnormal chloroplast and chromoplasts development (Dong et al. 2007; Qin et al. 2007). In common with PSY, PDS and ZDS co-exist in free inactive and membrane-bound active forms (Bonk et al. 1997; Mann et al. 2000; Lopez et al. 2008b; Joyard et al. 2009). The correct stereoisomer of each substrate is essential for the next enzyme catalysing step to carry out during the carotenoid biosynthesis. The first isomerisation step in the chromoplast is regulated by 15-*cis*-ζ-carotene isomerase (Z-ISO), which converts the product of the two sequential PDS reactions into an isomer that ZDS can utilise (Li et al. 2007). Many plants appear to have only a single copy of *Z-ISO*, but alternative transcripts were found in *Arabidopsis*, which lead to half of the protein products been non-functional and resulted in reduction of some carotenoids and elevation of β-carotene (Chen et al. 2010b). CRTISO, which converts the product of ZDS to all-*trans*-lycopene, is regulated through chromatin modification by methyltransferase SDG8. Reduced transcript abundance of *CRTISO* by the action of SDG8 in *Arabidopsis* alters the carotenoid profile (Cazzonelli et al. 2009). The discovery of the *tangerine* gene in tomato has also revealed the importance of CRTISO in production of all-*trans*-lycopene (Isaacson et al. 2002). Again, like the other enzymes in the

pathway, activity of CRTISO needs to be membrane bound for activity, which raises the possibility of multiple levels of regulation (Isaacson et al. 2004; Joyard et al. 2009).

*1.3.2.4 Lycopene to β- and α-carotenes*

The differential expression of the *LCYb* and *LCYe* genes determines the flux of the carotenoid biosynthetic pathway into either α-carotene or β-carotene. LCYb is encoded by a single gene in some plants such as *Arabidopsis*, rice, tomato and maize or by a small gene family in others such as some *Citrus* plants and three *LCYb* genes have been isolated from sweet orange (*Citrus sinensis* L.Osbeck) (Pecker et al. 1996; Lange & Ghassemian 2003; Bai et al. 2009; Chaudhary et al. 2010, Chen et al. 2010a). In red alga *Cyanidioschyzon merolae*, LCYb shows a higher fidelity for producing the β-ring than LCYe, whereas LCYe is less specific for producing either the ε-ring or β-ring (Cunningham et al. 2007). In maize, more than half of the natural genetic variation in lutein and β-carotene accumulation is regulated by the differential expression of the *LCYe* gene (Harjes et al. 2008). Gene silencing of the *LCYe* in *Brassica napus* results in increased β-carotene and total carotenoid content (Yu et al. 2008). It has been suggested that the formation of ε-ε−carotene is very unusual because of the rarity of LCYe/LCYe complex when compared with the LCYb/LCYb and LCYb/LCYe enzyme complexes (Cazzonelli & Pogson 2010). A recent proteomic study has revealed that LCYb is found in the membrane fraction like other members of the carotenoid biosynthetic pathway (Joyard et al. 2009).

Previous reports have suggested that down-regulation of the *LCYb* gene could cause accumulation of all e-ring containing carotenoids as seen in the delta mutant tomato (Ronen et al. 1999). The fruits of the delta mutant were found to accumulate higher level of *LCYe* transcript when compared with wild type, which caused a colour change from red to orange due to the accumulation of e-ring carotene (Ronen et al. 1999). The beta, old-gold (og) and old-gold-crimson ($og^c$) mutations have also been identified in tomato fruits and flowers as a change of the colour due to mutations of the *LCYb* gene. The og and $og^c$ mutations were caused by two distinct frame-shifts in the *LCYb* coding region. These caused a down-regulation of LCYb activity, and a changed flower and fruit colour due to increased accumulation of lycopene and a reduced level of β-carotene (Ronen et al. 2000). The beta mutation affects fruit colour only and is caused by an up-regulation of LCYb activity due to a mutation in the *LCYb* gene promoter (Ronen et al. 2000). Interestingly, the bate mutant

showed an increased accumulation of β-carotene in fruit only but not in flowers, which may indicate a differential regulation of carotenoid genes in chromoplasts of different tissue (Ronen et al. 2000). On the other hand, the red flesh papaya was due to an increased accumulation of lycopene compared with the yellow flesh fruit, but *LCYb* transcript abundance did not seem to increase (Skelton et al. 2006).

*1.3.2.5 Carotene to xanthophyll*

The hydroxylation step appears to be essential for the accumulation of carotenoids in chromoplasts. Yamamizo et al. (2010) found that the addition of the hydroxyl group enabled the carotenoids to be esterified, which was necessary for sequestration of the pigments in the chromoplasts of *Ipomoea* petals. In plants, the *CHY* genes are present as a small gene family composite of non-heme di-iron enzyme (BCH type) responsible for hydroxylating β-rings and cytochrome P450 enzyme (CYP97 type) hydroxylating β-rings and α-rings (Kim et al. 2009; Kim et al. 2010). The higher content of lutein in some inbred squash lines was also suggested to be related to their higher level of *CHYb* expression (Nakkanong et al. 2012). The over-expression of *CHYb* in *Arabidopsis* caused an increased production of zeaxanthin, neoxanthin and violaxanthin (Cho et al. 2008). The *Arabidopsis* CYP97 protein has been shown to be localised at the chloroplast envelope membrane (Joyard et al. 2009), and suggested by their protein structure BCH is also membrane bound (Joyard et al. 2009).

## 1.4 Use of VIGS to study carotenoid biosynthesis

Metabolite flux through pathways can be studied through knocking out or down the gene encoding for particular enzymes in the pathway. This can be achieved by different methods each of which has advantages and disadvantages. The loss of function approach was traditionally achieved at DNA or RNA level. Gene mutations at the DNA level can be randomly generated using the teratogenic chemicals such as ethyl methanesulfonate (EMS) (Silue et al. 2011) or by random insertion of T-DNA sequences (Tax & Vernon 2001). However, because of their random nature, large screening populations are required after the mutagenesis step and at least one generation of selfing is needed to generate homozygote lines. These procedures are time consuming and laborious. Post-transcriptional gene silencing approaches provide a more specific way of knocking down gene expression, where the gene of interest can be directly targeted without the need for screening of a large population. Post-

transcriptional gene silencing approaches utilise the RNAi machinery of the plant which recognises and degrades sequence-specific mRNAs and thereby prevents them serving as templates for protein synthesis. To silence genes in plants using RNAi, researchers initially used antisense approaches where they transformed plants with the targeted mRNA in an antisense orientation (Schuch 1991). More recently, researchers have increased the efficacy at which they can target the degradation of mRNA by transforming plants with the targeted sequence in a hairpin orientation (Stoutjesdijk et al. 2002; Wang & Wagner 2003).

There are a number of drawbacks to all of the above methods. The mutagenesis approaches require large populations for screening and the RNAi-based approaches require production of stably transformed plants which is both time consuming and often very difficult. Also, many of the transformation and regeneration systems have been established for model plants, but not for many other plant species. In addition, silencing of some essential genes can be lethal to the young plants. This may be particularly relevant to genes associated with carotenoid production as inhibition of carotenoid biosynthesis leads to photobleaching and reduced ABA production. For example effective silencing of *PDS* early in development has been shown to be lethal to young plants (Wang et al. 2005). Therefore, a method to silence the carotenoid genes that bypasses the early stages of plant development would be useful.

Virus-induced gene silencing (VIGS) is another RNAi-based method that circumvents many of the problems that arise through using stably transformed plants. For example, it allows silencing of genes in relatively established plant seedlings without the need to go through embryo or tissue culture stages. Phenotype of the plants can be seen in the same generation infected with virus. The VIGS procedure is not only a rapid method but also simple and overcomes the gene-dependent lethality that can be problematic in young plants. Transformation by VIGS is performed on established plant seedlings and no tissue culture steps are required. Many VIGS vectors have now been developed for different plant viruses which enable the technology to be used across many plant species including both eudicots and monocots (Purkayastha & Dasgupta 2009). Because of this the technology has been used increasingly for studying gene function in a wide variety of plants (Ratcliff et al. 2001; Liu et al. 2002; Wege et al. 2007; Godge et al. 2009; Pan et al. 2010; Purkayastha et al. 2010). It has also been successfully used to silence *PDS* in flowers of *Eschscholzia californica* (Wege et al., 2007). Given its many advantages, VIGS would seem an excellent technology for

allowing a deeper understanding of the regulation of one of the more fundamental and essential pathways in plant development, including that of carotenoid production.

### 1.4.1 The VIGS mechanism in plants

VIGS evolved in plants as a defence mechanism against virus invasion. During viral attack, plants produce small interfering RNAs (siRNAs) by cleaving double-stranded viral RNA that show sequence homology with the virus genome. The siRNA then direct RNA sequence-specific degradation of the viral genome (Figure. 1.3) (Lu et al. 2003; Benedito et al. 2004; Xie et al. 2004; Lim et al. 2008; Godge et al. 2009). The siRNA basis for VIGS was initially proposed after the discovery that plants which recovered from viral infection showed resistance to viruses that subsequently infected them if the nucleic acid sequence of the invading virus was similar to that of the previous virus (Inaba et al. 2011). The mechanism was also supported by the finding that suppressors of VIGS interact with the siRNA pathway (Takeda et al. 2005).

VIGS is initiated when plant protein recognise the double stranded RNA (dsRNA) produced by the viral genome as it replicates. The plant proteins that recognise the dsRNA are Dicer and Dicer-like proteins（DSLs) which process the dsRNA into 21-24 nucleotide primary siRNAs (Xie et al. 2004). These primary siRNAs are unwound into two single stranded siRNAs, which are the guide strand and the passenger strand. The siRNA guide strand binds to the plant RNA induced silencing complex (RISC) and guides the RISC to the viral RNA by sequence complementation. The RISC has RNase activity which it uses to degrade the viral RNA (Schwarz et al. 2002). The siRNA duplexes of either the guide strand or the passenger strand serve as the mobile silencing signals which are transported in the phloem of the plant and cause systemic silencing of the virus genes (Dunoyer 2010). A recent study has demonstrated that the small RNAs are transported from cell to cell and into the phloem *via* plasmodesmata (Liang et al. 2012). The study also showed that the silencing signal spread from cell to cell in the central stele of *Arabidopsis* (Liang et al. 2012). The primary siRNA-induced silencing is prolonged and more effective through the formation of secondary siRNA produced by siRNA signal amplification (Vaistij & Jones 2009). Signal amplification requires the action of the viral RNA dependent RNA polymerase (RdRP), which uses the single stranded siRNA and viral RNA as the primer and the template respectively, to make more

dsRNA. This newly created dsRNA is then digested to make more guide and passenger strand siRNAs.

In addition to silencing viruses through inducing viral RNA degradation, siRNAs can also silence viruses and suppress transposon activities by siRNA-induced methylation (Henderson & Jacobsen 2008). This phenomenon has also been observed in humans, as siRNA derived from the viral promoter sequence is able to induce promoter targeted gene silencing by DNA methylation (Lim et al. 2008). In plants, the methylation induced by the siRNA homolog to the repeated sequence is also associated with suppression of the transposon activities (Henderson & Jacobsen 2008).

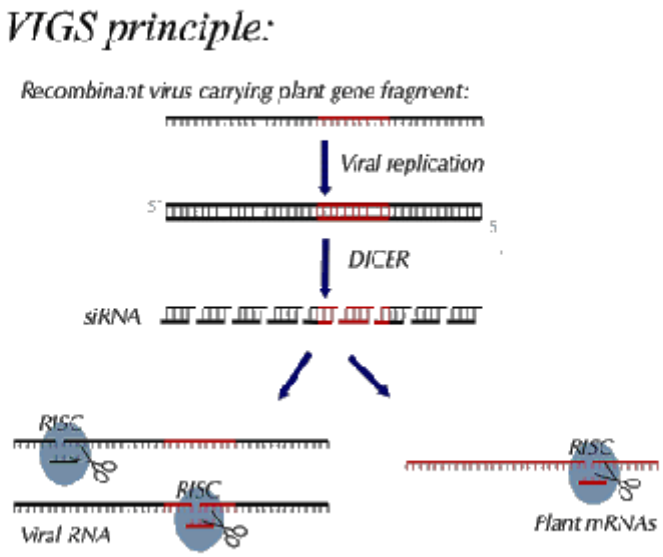



**Figure 1.3:** Schematic illustrating VIGS of plant mRNAs. The black lines represent the viral genetic material and red represents the inserted plant gene. The genetically engineered virus carrying the plant gene forms dsRNA through replication and the DICER protein recognises and cuts the dsRNA to generate siRNA complementary to both the viral gene and inserted plant gene. The siRNA-guided RISC causes degradation of the viral RNA and plant mRNA. (from Bach, 2008)

### 1.4.2 Structure and types of VIGS vectors

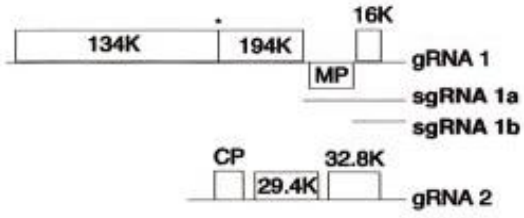
VIGS vectors have been designed to make it easy for researchers to clone the plant gene of interest into the viral sequence. Because of the limited host range of viruses, VIGS vectors have been constructed for many types of plant viruses, including the RNA virus *Tobacco rattle virus* (TRV), the DNA virus *Rice tungro bacilliform virus* (RTBV), the DNA satellite virus *Tomato leaf curl virus* (TLCV) and the RNA satellite virus *Satellite tobacco mosaic virus* (Voinnet 2001; Purkayastha & Dasgupta 2009).

VIGS vectors retain many of the basic components of the viral genome including the viral promoter, RdRP, coat protein, other components and a terminator sequence. In some cases, viral genes not needed for the success of the viral vector are removed, e.g., the nematode transmission genes of TRV RNA2 (Liu et al. 2002). A multiple cloning site is also typically added to simplify the cloning of the target plant gene sequence. In many cases, the CaMV 35S was used to drive the initial transcription of the viral RNA instead of its natural viral promoter, which will ensure the effective amplification of the vector both in vivo and in vitro. There are also several differences in the vectors depending on whether the vectors are based on RNA, DNA or satellite viruses. Vectors based on RNA viruses utilise the replication system of the RNA virus and replicate in the cytoplasm, whit those based on DNA viruses depend on the host replication system and replicate in the nucleolus. The ones based on satellite viruses need to be co-inoculated with their helper viruses (Godge et al. 2009; Purkayastha et al. 2010). The DNA viral vectors can be delivered to the plant by particle bombardment of the leaf tissue and the bombarded tissue extracts are then used to infect whole plants. Some earlier versions of the RNA virus based vectors needed to be reverse transcribed *in vitro* before delivery to the plants. Other RNA virus vectors such as *tobacco rattle virus* (TRV) are harboured in a binary vector backbone, which enables easy infection of plants *via Agrobacterium* infiltration (Ratcliff et al. 2001).

The VIGS system based on TRV has been successfully used in many plants including *Nicotiana tabacum, Eschscholzia californica* and *Papaver somniferum* (Hileman et al. 2005; Tafreshi et al. 2012). Two slightly different TRV-based vectors have been constructed for VIGS in plants (Ratcliff et al., 2001; Liu et al., 2002). The TRV based vectors developed by Liu et al were made from genetically engineered TRV single stranded RNAs RNA1 and RNA2. The TRV1 vector resembles RNA1 and contains the replicase proteins, the movement

protein and the cysteine-rich protein; while the TRV2 vector resembles RNA2 and contains the viral coat protein and multiple cloning site (MCS). Both TRV1 and TRV2 are driven by the duplicated CaMV 35S promoter (2X35S) and terminated by the nopaline synthase (NOS) terminator. Liu et al. (2002) also incorporated a self cleaving ribozyme site into TRV2 and located before NOS so that NOS is cleaved off after the first round of replication to mimic the natural situation. Both RNA1 and RNA2 sequences are separately harboured in binary vector backbones to enable *Agrobacterium*-mediated inoculation. Separate *Agrobacterium* cultures containing RNA1 and 2 are mixed just prior to inoculation into plants (Figure 1.4).

(A)




(B)

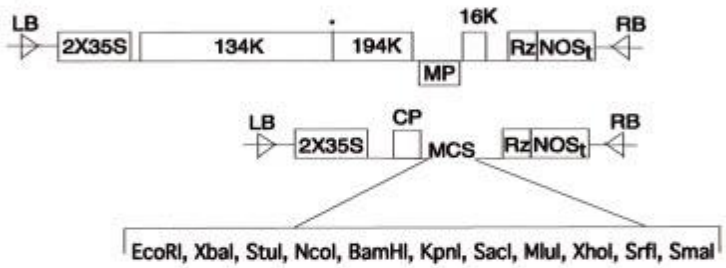



**Figure 1.4:** Schematic diagram illustrating TRV-based VIGS. (A) The original TRV genome. (B) The TRV based VIGS system. 134K= Helicase; 194K= RNA1 RNA dependent RNA polymerase; MP=movement protein; 16K= cysteine rich protein; CP=viral coat protein; 29.4K and 32.8K genes required for the transmission of the virus by nematodes; MCS= multiple cloning sites; 2X35S=duplicated CaMV 35S promoter; $NOS_t$= nopaline synthase terminator; Rz= ribozyme; LB=left border; RB=right border. Figure from Liu, (2002).

## 1.5 Carotenoid biosynthesis in orange *Eschscholzia californica* flowers

Carotenoids have been studied in the flowers of a range of plant species such as tomato, *Gentian*, lilies, marigold, *Oncidium, Ipomoea, Chrysanthemum* and daffodil (Zhu et al., 2010). The studies have predominantly focused on comparing carotenoid gene expression profiles with pigmentation patterns (Moehs et al. 2001; Yamamizo et al. 2010). In flowers of tomato, increased transcript abundance of the carotenoid biosynthetic genes was associated with increased carotenoid content (Giuliano et al. 1993). However just a few studies have looked at the end product feed-back regulation of the carotenoid biosynthetic pathway in the chromoplasts of flowers. The pioneering studies by Corona et al. (1996) and Al Balibi et al (1999), who induced accumulation of lycopene content in tobacco seedlings and daffodil flowers respectively by chemically blocking lycopene cyclase action with CPTA, appear to suggest that accumulation of lycopene, or reduction in metabolites downstream of lycopene does affect transcriptional regulation of the pathway. Also, Diretto et al (2007) reported targeted silencing of *CHYb* gene in potato tubers caused β-carotene and the total carotenoid content to increasely accumulate, which accompanied by up-regulation of the other carotenogenesis genes. However, clearly there is much still to learn about whether such feedback regulation is universal, and if so what are the metabolites that are responsible for such regulation.

To further understand the regulation of carotenoid biosynthesis in flower chromoplasts, I have used California poppy (*Eschscholzia californica*). *E. californica* has a short life cycle and a relatively small genome, which is only 6.5 times greater than that of *Arabidopsis.* This has enabled the species to become a model plant for eudicots for evolutionary developmental studies. An EST database of *E. californica* early flower development has also been made available publicly (Carlson et al. 2006). *E. californica* petals contain a variety of colours including orange, red and yellow due to their different carotenoid composition. Some flowers are white and this has been shown to be due to absence of carotenoids (Wakelin et al. 2003). Thus its naturally occurring carotenoid-based flower colour variation, short growth cycle and small genome size make *E. californica* a good choice for studying regulation of the carotenoid biosynthetic pathway.

## 1.6 Hypothesis and Aims:

**Hypothesis:** Silencing the expression of specific genes in the carotenoid biosynthetic pathway of the flower chromoplasts will lead to accumulation of specific metabolites in the pathway, which will subsequently feedback to up-regulate transcript abundance of part or of the whole pathway depending on the specific gene silenced.

**Aims:**

- To use TRV-based VIGS to silence targeted carotenogenesis gene transcripts in *E. californica* petals.

- To determine whether specific carotenogenesis metabolites increasely accumulate in response to targeted gene silencing of the pathway.

- To test whether these changes in carotenoid metabolites feedback to alter transcript abundance of the carotenogenesis pathway.

# CHAPTER TWO
# MATERIALS AND METHODS

## 2.1 Chemicals, buffers, solutions and media

All chemicals, reagents and solvents were sourced from Sigma (Sigma-Aldrich Co. LLC. St. Louis, MO, USA) Merck (Merck KGaA, Darmstadt, Germany) Invitrogen **(**Life Technologies New Zealand Limited, 18-24 Botha Road Penrose, Auckland 1006, New Zealand) and Roche (Hoffmann-La Roche Ltd. Basel, Switzerland) unless otherwise stated. MilliQ water was used to make buffers and solutions. Autoclaved milliQ water was used when the solution was not to be autoclaved after making. Luria-Bertani (LB) broth and agar plates for bacterial culture and selection were autoclaved following preparation. Appropriate antibiotics were added to LB broth after cooling of it to room temperature and to agar after cooling to 50 °C. Recipes for all buffers, solutions, media and antibiotic stocks used are listed in Appendix 1.

## 2.2 Plant material and growth conditions

*Eschscholzia californica* (Californian Poppy) seeds were purchased from Gardenstuff (Gardenstuff Ltd. Prebbleton New Zealand) and grown in the PC2 containment glasshouse facility at Plant & Food Research, Palmerston North. Seeds were germinated in Plant & Food pH mix (Appendix 1) on a heat pad with intermittent misting for 7 days. After germination, the seedlings were moved to standard benches in the temperature-controlled greenhouse to grow for seven weeks. The greenhouse was set to heat when the temperature is below 16 °C and ventilate when above 22 °C. Every ten seven-week-old seedlings were transplanted to a tray (60 cm x 30 cm x 10 cm) and moved to a Viral House unit within the PC2 containment glasshouse for VIGS treatment. Ten trays were prepared in total for a single replicate of the experiment. Experiments were conducted between August 2011 and January 2012 with no supplemental lighting. The natural day length during this period was approximately 10.5-14 h. The temperature of the Viral House unit was maintained by air conditioning units, which were set to 22 °C.

## 2.3 General microorganism growth conditions

*Escherichia coli* strain Novablue (NB) and *Agrobacterium tumefaciens* strain GV3101 (GV3101) were employed in this study. NB was used for general lab manipulations including cloning and plasmid amplification. GV3101 was used for transferring T-DNA containing viral vector and plant sequences into plants. Both NB and GV3101 were cultured in LB broth and on LB agar plates with appropriate antibiotics. Liquid cultures were shaken at 250 rpm during incubation. NB was grown at 37 °C overnight and GV3101 was grown at 28 °C for 1-2 days.

## 2.4 Isolation of candidate sequences of carotenoid biosynthetic genes

### 2.4.1 Polymerase chain reaction (PCR)

Putative carotenoid biosynthetic gene sequences were isolated from *E. californica* petal cDNA used the polymerase chain reaction (PCR). *E. californica* petal cDNA was provided by Philippa Barrell (Plant & Food Research, Lincoln), and the primers listed in Table 2.1 were used to amplify candidate gene sequences including phytoene synthase (*EcaPSY*), phytoene desaturase (*EcaPDS*), ζ-Carotene desaturase (*EcaZDS*), lycopene β-cyclase (*EcaLCYb*), lycopene ε-cyclase (*EcaLCYe*), β-ring hydroxylase (*EcaCHYb*) and zeaxanthin epoxidase (*EcaZEP*). The primers were selected from previous literatures which showed successful amplification of the above genes. The FastStart High Fidelity PCR System (Roche) was used to amplify all PCR products, using either an Eppendorf (Eppendrof South Pacific Pty. Ltd. NSW. Australia) or Bio-Rad (Bio-Rad Laboratories (New Zealand) Pty. Ltd. Auckland) PCR thermocycler. The set up of PCR mix and reaction conditions is shown in below.

**PCR mix:**

34.5 μL of water

5 μL of 10x buffer with $MgCl_2$ (2 mM)

3 μL of DMSO (6% v/v)

1 μL of 10 mM dNTP (200 μM)

2 μL of 10 μM forward/sense primer (0.4 μM)

2 μL of 10 μM reverse/antisense primer (0.4 μM)

2 μL=100 ng template cDNA

0.5 μL of Faststart Taq (2.5 U)

50 μL Total volume

**PCR conditions:**

95 °C for 2 min

95 °C for 30 sec

50-59.2 °C for 30 sec

40 cycles

72 °C for 1 min

(1Kb/min extension)

72 °C for 5 min

**Table 2.1:**

Degenerate primers, melting temperature (TM) and expected product sizes for the carotenoid biosynthetic gene isolation as indicated

| Gene | Primer | Sequence | TM °C | Expected size kb | Reference |
|---|---|---|---|---|---|
| *PDS* | PDS-F1 | CGGTCTAGAGCCACTMAACTTYATAAAC | 57 | 0.4 | Wege 2007 |
| | PDS-R1 | CGGGACTCCTTCAGTTTTCTGTGAAACC | | | Wege 2007 |
| *PSY* | CmPSY-S | GAAGGAATGMGWATGGACTT | 51.8 | 0.48 | Ikoma 2001 |
| | CmPSY-AS | GCTTCRATCTCRTCYAGTAT | | | Ikoma 2001 |
| *ZDS* | PmZDS-S | TAYGCYYTNGGWTTYATWGACTG | 55 | 0.85 | Kita 2007 |
| | PmZDS-AS | GCTCCTTCCATRCTRTCDATGTARTC | | | Kita 2007 |
| *LCYb* | PmLCYb-S | AAYAAYTAYGGWGTTTGGWDGATGA | 49 | 0.9 | Kita 2007 |
| | PmLCYb-AS | YARRAANCCDTGCCA | | | Kita 2007 |
| *LCYe* | PmLCYe-S | ACTGTTGCWTCKGGRGCAGC | 55 | 0.7 | Kita 2007 |
| | PmLCYe-AS | CCACATCCAHKTTGGMAMDCGRAA | | | Kita 2007 |
| *CHYb* | PmHYb-S | TGGMGAGRAAGMRATCSGAGAGG | 55 | 0.56 | Kita 2007 |
| | PmHYb-AS | TCCTTRGGWCCNARRAASARBCCATA | | | Kita 2007 |
| *ZEP* | PmZEP-S | GCTGCTTTGGAAGCYATTGAT | 54 | 1.48 | Kita 2007 |
| | PmZEP-AS | CKAAAMCGMGCRGGAAARTT | | | Kita 2007 |

### 2.4.2 Gel electrophoresis

PCR and other DNA fragments were separated by gel electrophoresis. Aliquots (50 µL) of PCR product or 5 µL digested DNA was mixed with the appropriate volume of 10x DNA loading dye (Appendix 1). The mixture was loaded into a 1% (w/v) TBE agarose gel containing 0.2 µg/mL ethidium bromide (Appendix 1) and resolved at 100 V in 1x TBE buffer for *ca* 30 mins. The ethidium stained DNA was visualised with UV light using a transilluminator (Bio-Rad) and photographed using a gel-doc system (Bio-Rad).

### 2.4.3 Purification of PCR products

The gel areas containing the PCR products of interest were excised using a sterile scalpel blade and DNA recovered from the gel using the Zymoclean™ Gel DNA Recovery kit (Zymo Research Corporation, Irvine CA. USA), following the manufacturer's protocol. The concentrations of the purified DNA fragments were measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington DE. USA).

### 2.4.4 TA-cloning of PCR products into the pGEM®-T Easy vector

Putative carotenoid gene fragments generated by PCR were TA-cloned into the pGEM®-T Easy vector (Promega BioSciences LLC San Luis Obispo,CA,USA) for sequencing analysis. TA-cloning was possible because FastStart Taq DNA Polymerase (Roche) has terminal transferase activity which results in an adenosine residue being incorporated onto the 3´-end of the PCR fragment and this provides a sticky end to bind with the overhanging thymine residue of the pGEM®-T Easy vector. Ligation of the PCR insert into the vector was carried out using the Rapid DNA Ligation Kit (Roche) according to the manufacturer's instructions. Ligations were performed in a total volume of 10-12 µL with an insert to vector ratio of 3:1. The ligation mix was incubated at room temperature from 2 to 16 h. The amount of insert needed to get the 3:1 ratio was calculated according to the equation.

$$\frac{\text{50 -100 ng vector x Kb insert}}{\text{Kb vector}} \times 3 = \text{insert quantity}$$

### 2.4.5 *E.coli* transformation

The ligation mix containing the pGEM®-T Easy vectors potentially with inserts were transformed into *Escherichia coli* strain Novablue (NB) competent cells (gifted by the molecular lab Plant & Food Research, Palmerston North) for convenient storage and multiplication of the PCR-amplified products. Approximately 5-6 μL of the ligation reaction was very gently mixed with 50 μL of ice-thawed NB competent cells. The cells were then incubated on ice for 15 min, heat shocked at 42°C for 45 sec and incubated on ice further for 5 min. The transformed cells were spread onto LB agar plates containing 100 μg/mL ampicillin and surface pre-coated with 40 μL of 20 mg/mL bromo-chloro-indolyl-galactopyranoside (X-Gal) and 4 μL of 200 mg/mL isopropyl β-D-1-thiogalactopyranoside (IPTG) to enable blue white screening of the resultant colonies, and then incubated overnight at 37 °C. Blue/white screening is possible because the TA-cloning site has been placed within the *lacZ* gene and insertion of the sequence into the *lacZ* gene disrupts the production of β-galactosidase necessary to cleave X-Gal into the blue coloured product. Therefore a white colony is suggestive of an insert being successfully cloned into the vector, whereas a blue colony is not. It however also should be noted that pale blue colonies may sometimes contain insert if the insert has gone in-frame with the *lacZ* gene as read through can still sometimes occur to make a functional β-galactosidase. Yet, white colonies were picked from the plate and grown overnight at 250 rpm in 3 mL LB liquid media containing 100 mg/mL ampicillin in a 37 °C shaking incubator.

### 2.4.6 Screening for positive clones

Plasmids were purified from the overnight cultures by an alkaline lysis plasmid miniprep procedure (Section 2.4.6.1). Plasmids were then digested with the appropriate restriction enzymes (Section 2.4.6.2) and the digested products resolved on an agarose gel (Section 2.4.2) and the expected digestion pattern used to confirm the presence of an insert. The pGEM®-T Easy clones with PCR product insert have been digested with *Eco*RI in Buffer H at 37 °C.

*2.4.6.1 Alkaline lysis plasmid miniprep procedure*

Cells were collected from 1.5 mL of the 3 mL overnight NB cultures by centrifugation at 21913 x *g* at room temperature. The cell pellet was resuspended in 200 µL of Solution I (Appendix 1), immediately lysed by adding 400 µl of Solution II (Appendix 1) and mixing gently by inversion until the solution clears. The lysed cells were then neutralised by adding 300 µL of Solution III (Appendix 1) and the cellular debris immediately removed by centrifugation at 21 913 x *g* at 4 °C for 10 min. The supernatant was transferred to a fresh tube and the plasmid DNA precipitated with 700 µL of isopropanol. The DNA pellet was collected by centrifugation at 21913 x *g* at 4 C° for 10 min washed once with 70% ethanol, then air-dried and resuspended in 30 µL of sterile water.

*2.4.6.2 Restriction enzyme digestion*

Aliquots of plasmid, typically 1 µL, obtained from the alkaline lysis procedure were digested with 2 U of the selected restriction-endonuclease in the appropriate buffer in a total volume of 10 µL at the suggested temperature. Digestion was carried out for no longer than 2 hours to avoid star activity, which refers to redaction of specificity of restriction-endonuclease under non-standard reaction conditions.

2.4.7 Sequencing

The positive clones identified by restriction digestion were sent for sequencing at the Allan Wilson Centre Genome Service, Massey University (Palmerston North). Plasmids sent for sequencing were column purified (see Section 2.4.3) and their concentration determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The purified plasmid was diluted to 32 ng/µL (450 ng in 14 µL) as required by the sequencing centre. All clones were sequenced from both directions using the T7 (forward) primer and SP6 (reverse) primer.

The putative identity of the cloned PCR products was obtained by sequence comparison with GenBank sequences using the Basic Local Alignment Search Tool (BLAST) algorithms blastn and blastx (Altschul et al., 1997), (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To obtain larger fragments of the genes, sequences were sent to Dr Mark Fiers (Plant & Food Research, Lincoln) who used them to search *E. californica* 454 sequences obtained from pooled petal tissues of two different genotypes (orange and white) at two different stages of development (very immature [green] and well before anthesis [at green orange transition] ).

## 2.5 Cloning of carotenoid biosynthetic genes into the TRV2 vector

### 2.5.1 Selection of region of carotenoid biosynthetic gene for insertion into TRV2

Selection of the portion of the carotenoid biosynthetic gene for insertion into TRV2 was prudent. In addition to there being a need for sufficient length of sequence for inducing good silencing of the gene in the plant, there is also a requirement for this region to be outside the cloned region that could be used in qRT-PCR to assess the efficacy of silencing. Because of this, the primer sites required for reliable qRT-PCR were first identified in the sequences, and then primers were designed to amplify a *ca* 200-700 bp region that would be used for insertion into TRV2, with the proviso that at least one of the qRT-PCR primers fell outside this region. Primers were designed using PrimerSelect (DNASTAR, Lasergene) to the carotenoid biosynthetic gene sequences obtained either from the degenerate PCR or from the *E. californica* 454 sequence database. Primers used for cloning into TRV2 had *Sac*I and *Xba*I restriction-endonuclease sites added to the 5′-end of the sense and antisense primers respectively (Table 2.2, Appendix 2).

**Table 2.2:**

Primers used for amplifying sequences of the carotenoid biosynthetic genes for insertion into TRV2.

| Gene | Primer | Sequence | TM °C | Expected size bp |
|---|---|---|---|---|
| *EcaPSY* | CepsyAS445*Sac* | GGCGAGCTCATACAGTAACAAAGATGCCCACAC | 57 | 284 |
| | CepsyS161*Xba* | GGCTCTAGATATAGTGCTGCTTTGGCGTTAG | | |
| *EcaPDS* | PDS-F1 | CGGTCTAGAGCCACTMAACTTYATAAACC | 57 | 407 |
| | PDS-R1 | CGGGACTCCTTCAGTTTTCTGTGAAACC | | |
| *EcaZDS* | CeZDS-AS559*Sac* | GGCGAGCTCCGGAGACGTGAGTGCGAGAT | 57 | 384 |
| | CeZDS-S175 *Xba* | GGCTCTAGATGGCAGGTTTCATCTCAGGTG | | |
| *EcaLCYb* | CeLyCyclAS725*Sac* | GGCGAGCTCTTTGATTGTTGATGCAAGTGGGTTTC | 58 | 450 |
| | CeLyCyclS275*Xba* | GGCTCTAGATGAAGGGTGAACCAATCCTGCACT | | |
| *EcaHCYb* | CHYbS209Xba | GGCTCTAGAGAGTTTCCATGGCGGCTGGGT | 55 | 683 |
| | CHYbAS892Sac | GGCGAGCTCGTCCAGCACCAAAACAGAGACCAGG | | |
| *EcaZEP* | CeZEP-AS507*Sac* | GCCGAGCTCCCCACATCCGAAGAAACAAAGTAT | 54 | 451 |
| | CeZEP-S56*Xba* | GGCTCTAGAAAAGCTGGTTGTATTACTGGTGAT | | |

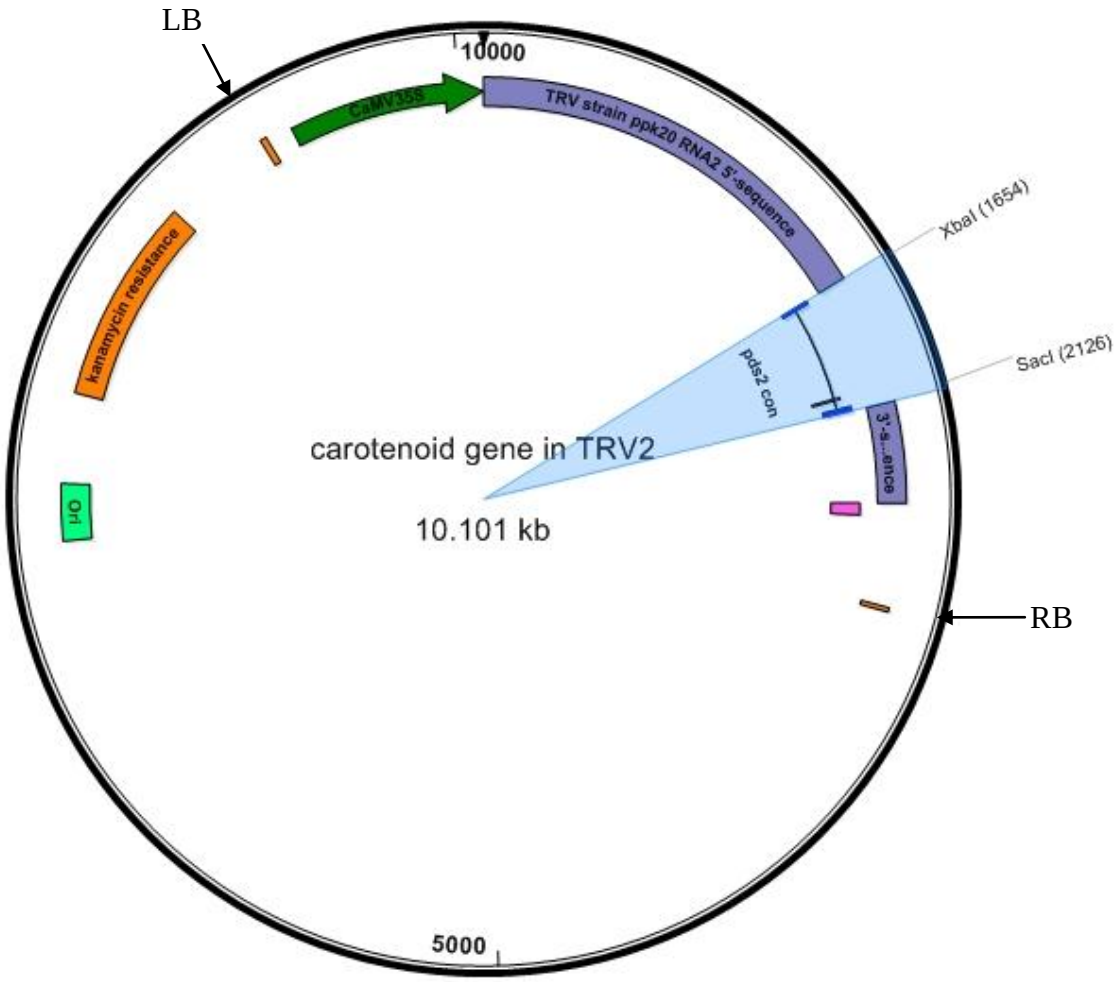



**Figure 2.1:** Representative map of the TRV2 vector containing a carotenoid biosynthetic gene fragment. Each carotenoid gene was inserted into the TRV2 vector between the *Sac*I and *Xba*I restriction sites. Ori, origin of replication; CaMV35S, constitutive promoter from Cauliflower Mosaic Virus; purple region, TRV2 sequence containing a full-length cDNA of PPK20 RNA 2 the *Sac*I and *Xba*I restriction sites; blue sector, the inserted carotenoid gene fragment. RB and LB are the right and the left borders.

### 2.5.2 Cloning of carotenoid biosynthetic gene fragments into TRV2

Regions of the carotenoid biosynthetic gene sequences to be inserted into TRV2 were amplified by PCR using primers listed in Table 2.2 and *E. californica* cDNA as the template following the procedure described in 2.4.1. The resultant products were resolved by gel electrophoresis (Section 2.4.2), the fragments of expected size cut from the gel and purified (Section 2.4.3). Both the TRV2 vector and the purified PCR products were digested with *Xba*I and *Sac*I in Buffer H (Section 2.4.6.2) then resolved by gel electrophoresis (Section 2.4.2) and purified (Section 2.4.3) in readiness for ligation. The inserts were ligated into the TRV2 vector (Figure 2.1) using the Rapid DNA Ligation Kit (Roche) according to the manufacturer's instructions (Section 2.4.4). The resultant TRV2 vector with insert was transformed into NB (Section 2.4.5) and colonies selected on LB plates containing 60 μg/mL kanamycin. The positive colonies were screened according to Section 2.4.6 using *Xba*I and *Sac*I as the endonucleases. The resultant positive TRV2 vectors were electroporated into GV3101.

### 2.5.3 GV3101 transformation

Electrocompetent cells of GV3101 were transformed with TRV2 constructs by electroporation. Plasmid (1 μL) isolated by the alkaline lysis plasmid miniprep procedure (Section 2.4.6.1) was added to 20 μL of GV3101 competent cells (gifted by molecular lab Plant & Food Research, PN, following the procedure of McCormac et al. 1998) just-thawed on ice in a microfuge tube and transferred to a pre-chilled electroporation cuvette. Cells were electroporated using a Cell-Porator Electroporation System (Life Technologies Corporation, Auckland, New Zealand) following the instructions of the manufacturer. LB broth (1 mL) was added to the cuvette immediately after electroporation and cells were allowed to recover by transferring to a 14 mL bacterial culture tube which was shaken at 28 °C for 3 h at 250 rpm. The transformed cells were selected by spreading on LB plates containing 20 μg/mL gentamicin (selection for helper plasmid), 60 μg/mL kanamycin (selection for pTRV2) and 50 μg/mL rifampicin (selection for GV3101).

2.5.4 Identification of positively transformed GV3101 cells

Positively transformed GV3101 colonies were not able to be clearly identified by restriction digestion of their plasmids which had been isolated using the alkaline lysis procedure (Section 2.4.6.1). Because of this, the plasmids from the putative positive GV3101 colonies were retransformed back into NB to enable confirmation of the insert by restriction digestion analysis. To do this, individual GV3101 colonies were picked and grown for 2 days at 28°C in a 3 mL LB culture under the selection (Section 2.5.3). The cells were then collected by centrifugation at 21913 x *g* for 1 min and plasmids isolated using the alkaline lysis plasmid miniprep procedure (Section 2.4.6.1). The purified plasmids were transformed into NB cells and putative transformants selected on LB plates containing 60 µg/mL kanamycin (Section 2.4.5). To confirm that the colonies were positive, plasmids were isolated using the alkaline lysis procedure (Section 2.4.6) digested with *Xba*I and *Sac*I and the resultant fragments resolved in an agarose gel to identify the presence of the expected size insert (Section 2.4.2). The positive GV3101 colonies were then stored at 80 °C as bead stocks using Microbank™ (PRO-LAB DIAGNOSTICS, ON, L4B 1K3 Canada) following the manufacturer's instructions.

## 2.6 Agro-inoculation of plants with VIGS vectors

2.6.1 Preparation of GV3101 for plant inoculation

*E. californica* plants were infected with a 1:1 mix of TRV1-containing (Section 1.4.2, gifted by molecular lab Plant & Food Research, PN, prepared following the procedure of Section 2.5.3) and TRV2-containing GV3101 cultures. Cultures were prepared for plant inoculation according to the following procedures. A microbead containing the appropriate GV3101colony (Section 2.5.4) was used to inoculate a 3 mL LB broth containing 20 µg/mL gentamicin, 60 µg/mL kanamycin and 50 µg/mL rifampicin. Cultures were held for 2 days in a 28°C shaking incubator set to shake at 250 rpm. Next, 25 µL of the starting culture was used to inoculate a 25 mL broth, which contained 10 mM MES and 20 µM acetosyringone in addition to the same antibiotics used in the starting culture. A 25 mL LB broth for the TRV1 vector was also prepared using the same culture media as for the TRV2 vector. Both cultures were shaken at 250 rpm overnight at 28 °C. The cells were transferred to 50 ml FALCON

tubes, which were then centrifuged at 5311 x *g* for 15 min at 4 °C to pellet the cells. The cell pellets were finally resuspended in 25 mL of infiltration buffer (Appendix 1) and incubated at room temperature for 4 h. The TRV1-containing and TRV2-containing GV3101 cultures in infiltration buffer (inoculum) were then mixed 1:1 and used to inoculate the plants.

### 2.6.2 Agro-inoculation of plants

*E. californica* plants were inoculated by both agro-infiltration and agro-drench seven weeks after germination. Ten replicate plants were inoculated with each TRV2 construct. The primary infiltration was carried out by injecting 1 mL of inoculum into the base of the petioles and the meristem region at the centre of the plants using a syringe with needle. The next day, the infiltration procedure was repeated using the inoculum from the previous day that had been stored at room temperature. On the fourth day and every second day thereafter, 1 mL of the remaining inoculum was used to drench the soil at the base of the plant. This was repeated until the leaves of TRV-*pds* plants as the marker of silencing showed signs of photobleaching. All inoculations were carried out in the light in growth chambers set to 22 °C and plants were incubated under the same conditions. The experiment was repeated to give 2 x 10 plants for each treatment.

## 2.7 Phenotype observation

Changes in overall plant development and colouration of new leaves and flowers were recorded. The colour changes were first observed under white light, the same samples were assessed again under long wave ultraviolet light if no colour difference was shown under white light. Flower colour was observed at anthesis stage.

## 2.8 Sample collection

All flowers used for analysis were harvested at anthesis between 11am-12am daily, photo documented, frozen in liquid nitrogen and stored at -80 °C. The flowers showing the most consistent VIGS phenotype from 4-5 different plants in the set of 10 were pooled for each biological replicate. The two biological replicates that were collected, ground under liquid nitrogen, freeze dried for 4 days and stored at -20 °C.

## 2.9 Gene profiling

### 2.9.1 RNA isolation

Total RNA was isolated from 30 mg of freeze dried tissue using a modified hot borate RNA extraction method (Wan and Wilkins 1994). The powdered tissue was mixed with 0.5 mL of preheated Hot Borate total RNA extraction buffer (Appendix 1) for 10 min at 80 °C. After mixing by vortexing for 30 sec, and then 1.9 µL of proteinase K (20 mg/mL) (A&A Biotechnology, Al. Zwycieswa, Poland) was immediately added and the mixture then incubated in the thermomixer Comfort (Eppendorf) at 42 °C for 1.5 h. After incubation, 40 µL of 2 M KCl was added to the sample and mixed gently, before incubation on ice for 30 min after which the supernatant was separated from the cellular debris by centrifugation at 21913 x *g* for 30 min at 4 °C. The supernatant was transferred to a fresh tube, mixed with an equal volume (0.5 mL) of 4 M LiCl and precipitated at 4 °C overnight. The precipitated RNA was collected by centrifugation at 21913 x *g* for 30 min at 4 °C, and the pellet was washed twice with 1 mL of 2 M LiCl. The RNA pellet was resuspended in 400 µL of 10 mM Tris-HCl pH 7.5 and 40 µL of 3 M potassium acetate. Non-dissolvable debris was removed from the resuspension by centrifugation at 21913 x *g* for 10 min at 4 °C, the suspended RNA was re-precipitated with 1 mL of absolute ethanol at -80 °C overnight. The precipitated RNA was collected by centrifugation at 21913 x *g* for 30 min at 4 °C. The RNA pellet was washed with 0.5 mL of 70% ethanol, air dried and resuspended in 30 µL of sterile water. RNA concentrations in the samples were measured with the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and the quality of the RNA samples assured by determining the absorbance ratios at 260/280 and 260/230. An $OD_{260}/OD_{280}$ ratio of 1.8-2 is reflective of low protein contamination and a $OD_{260}/OD_{230}$ ratio of >1.8 indicates low polysaccharide contamination. The RNA integrity was checked by 1.2% (w/v) MOPS agarose denaturing gel electrophoresis (Appendix 1).

### 2.9.2 Real-time quantitative PCR(qRT-PCR) primer design

Real time quantitative PCR was carried out on cDNA synthesised from RNA isolated from petals of TRV infiltrated plants. The qRT-PCR primers listed in Table 2.3 were designed to the carotenoid gene sequence information obtained from the *E.californica* 454 sequencing

database.PrimerQuest(http://eu.idtdna.com/Scitools/Applications/PrimerQuest/Default.aspx?SequenceWarning=True) and PrimerSelect (DNASTAR, Lasergene) programme were used for the design of primers. Primer locations are shown in Appendix 2.

**Table 2.3:**

Primers used in qRT-PCR

| Primer | Sequence | Gene |
|---|---|---|
| KSDT | 5´-CGGTACCGATAAGCTTGATTTTTTTTTTTTTTTTTV-3´ | RT |
| 18S | GAGCTGGAATTACCGCGGCT | 18S ribosomal RNA |
| ECaPDS853 S | AGTACGGTTTGCCATCGGACTCTT | *EcaPDS* |
| ECaPDS1043 AS | TGCACTGCATTGAAAGCTCGTCTG | *EcaPDS* |
| CePsyS130 | ACCAGAATCTCAAGCAACGACGGA | *EcaPSY* |
| CePsyAS273 | TGTGCTAGCTCATCCTGTGGTAGA | *EcaPSY* |
| CeZDS-S79 | TGCCACTAAGACAGAGGCTTCGTT | *EcaZDS* |
| CeZDS-AS194 | ACCTGAGATGAAACCTGCCACCTT | *EcaZDS* |
| CeLyCyclS182 | CCCTCTAATCATCCTAGTGGACCCTAAAC | *EcaLCYb* |
| CeLyCyclAS306 | ATGGTGCAAGTGCAGGATTGGTTC | *EcaLCYb* |
| BcarS861ECa | CCTCTTTCCTGGTCTCTGTTTTGGTG | *EcaCHYb* |
| BcarAS980ECa | ACATTAGCAATGGGTCCGACTGGA | *EcaCHYb* |
| CeZEPS1000 | AGTTACGAATACCGCACCCAGGAA | *EcaZEP* |
| CeZEPAS1188 | GCTCTAATGCATCATCATCTTCAAACCA | *EcaZEP* |
| ActS467ECa | ACCACTGAGCACGATGTTTCCGTA | *EcaACTIN* |
| ActAS622ECa | ATTGGAGCAGAAAGGTTCCGTTGC | *EcaACTIN* |
| EF486SECa | TCACCCTTGGTGTCAAGCAGATGA | *EcaELONGATION FACTOR* |
| EF665ASECa | TGTTGTCACCCTCAAACCCAGAGA | *EcaELONGATION FACTOR* |

2.9.3 qRT-PCR

All RNA samples to be used in qRT-PCR were diluted to 120 ng/µL in a total volume of 10 µL. An aliquot (1 µL) was then used to reconfirm the concentration of RNA using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and this measurement would also be used as an additional reference for determining how the transcript abundance of the reference genes (*ACTIN*, *ELONGATION FACTOR*) varied in the different tissues analysed in the qRT-PCR. An aliquot of the diluted RNA (8 µL), corresponding to *ca* 1 µg RNA, was then DNase treated using DNase1 Amplification Grade (Invitrogen) and then reverse transcribed using Transcriptor Reverse Transcriptase (Roche) according to the manufacturer's instructions. Both the KSDT and 18S primers (Table 2.3) were used to prime the synthesis of the cDNA, and after synthesis, the cDNA was diluted 20-fold to 400 µL total volume with sterile water.

qRT-PCR reactions were set up for a single using reaction 2.5 µL cDNA, 2.5 µL primer mix (0.5 µM sense and antisense primers final concentration), 5 µL LightCycler® 480 SYBR Green 1 Master mix (Roche) in total volume of 10 µL. The reactions were setup in triplicate using a CAS1200$^{TM}$ precision liquid handling system (Corbett Life Science, Concorde, NSW, Australia). To help minimise variation in qRT-PCR, the sense and antisense primers were premixed into a stock from which 2.5 µL was used in each reaction (to give final concentration of 0.5 µM in 10 µL qRT-PCR). The stock was aliquoted into smaller volumes to minimize effects of freeze thaw cycles. The recipe for a single reaction is shown below as well as cycling conditions used in the qRT-PCR. The qRT-PCR was carried out in a Corbett Life Science Rotor-Gene 3000 PCR machine and the results analysed using Rotor Gene 6000 series software and the method of Pfaffl et al. (2004). The relative abundance of carotenoid gene transcripts was normalised against the reference genes *ACTIN*, and *ELONGATION FACTOR*.

| | | |
|---|---|---|
| 0.5 µL of 10 µM Sense primer | Cycling conditions: | |
| 0.5 µL of 10 µM Antisense primer | 95 °C 10 min | |
| 1.5 µL of water | 95 °C 10 s | |
| 5 µL of SYBR Green Master Mix | 60 °C 15 s | X40 |
| 2.5 µL of 20 x diluted cDNA | 72 °C 20 s | |
| 10 µL Total | | |

## 2.10 Metabolite profiling

### 2.10.1 Carotenoid extraction, partition and saponification

Carotenoids were extracted from 25 mg of freeze dried flower petal tissue using 1 mL of carotenoid extraction buffer [acetone:methanol, 7:3] with 200 mg/mL $CaCO_3$ (Appendix 1). The mixture was then vortexed and held at room temperature for 1 h. The insoluble matter was pelleted and the supernatant removed to a fresh foil-covered 15 mL tube and held at room temperature, while the pellet was then re-extracted four more times using carotenoid extraction buffer without $CaCO_3$. Three of the incubation times were again for 1 h while one extended to overnight.

The five extracted supernatants were combined to give a total extractant volume of *ca* 4.6 mL and then partitioned with diethyl ether according to the following procedure. A 2 mL aliquot of 10% NaCl (w/v) and then 2 mL of diethyl ether was sequentially added to the extractant with inversion after each addition. If the solution did not partition, then 1 mL of water was also added to aid separation. The top acetone:methanol fraction of the partition was collected to a glass tube. The partition procedure was repeated twice more or until the acetone:methanol phase was colourless. The combined diethyl ether fractions were then dried under $O_2$-free $N_2$ and the carotenoids dissolved in 2 mL of 0.8% BHT:acetone (w/v) as described by Ampowmah-Dwamena et al. (2009). The total carotenoid extractant for each petal sample was stored in the dark at 4 °C.

An aliquot (400 µL) of the 2 mL total carotenoid extractant was then saponified by mixing with 10% potassium hydroxide:methanol (w/v). The mixture was incubated at 4 °C overnight and then cleaned up by re-partitioning as described above, that then the combined diethyl ether fraction were re-dissolved in 400 µL of 0.8% BHT:acetone (w/v).

### 2.10.2 Spectrophotometric determination of total carotenoid concentration

The total carotenoid concentration in the carotenoid extractant was determined spectrophotometrically (Jasco V-530 UV/Vis spectrophotometer, Jasco, Tokyo, Japan) using chloroform diluted samples. Aliquots of 5 µL or 10 µL of the carotenoid extractants were

diluted with 995 µL or 990 µL respectively of chloroform and read against a chloroform blank. Readings were taken at 480nm, 648 nm and 666nm. The total carotenoid concentration of the extractant was calculated using Wellburn's equation (Wellburn 1994).

Total carotenoid $Cx+c=(1000A_{480}-1.42Ca-46.09Cb)/202$
=µg/mL x Dilution factor
=µg/mL x (volume of extract) ml =µg x 1000
= mg/weight (tissue) g DW
=mg/gDW

Cx +c=Total carotenoid
Ca=Chlorophyll a=$10.91A_{666}-1.2A_{648}$
Cb =chlorophyll b=$16.36A_{648}-4.5A_{666}$

**Table 2.4:**

Elution gradients of HPLC (A) and LCMS (B) analyses

A. HPLC

| Solvent / Time (min) | A MeOH | B $H_2O$:MeOH (20:80) containing 0.2% ammonium acetate | C MTBE | Flow rate (mL/min) |
|---|---|---|---|---|
| 0-2 | 95% | 5% | N/A | 1 |
| 2-10 | 80% | 5% | 15% | 1 |
| 30 | 30% | 5% | 65% | 1 |
| 40 | 25% | 5% | 70% | 1 |
| 45 | 95% | 5% | N/A | 1 |

B. LCMS

| Solvent / Time (min) | A MTBE | B MeOH | Flow rate (µL/min) |
|---|---|---|---|
| 0 | 5% | 95% | 200 |
| 33 | 62.7% | 37.3% | 200 |
| 33.5-36 | 90% | 10% | 300 |
| 36.5-39.5 | 5% | 95% | 300 |

### 3.10.3 HPLC analysis of petal carotenoid profile

Samples (50 µL) were separated by HPLC using a Dionex Ultimate 3000 solvent delivery system fitted with a YMC RP C30 column (5 µm, 250 x 4.6 mm), coupled to a 20 x 4.6 C30 guard column (YMC Inc. Wilmington, North Carolina, USA) and a Dionex 3000 PDA detector. The column elution rate was 1.0 mL/min and with a column temperature of 25 $^{o}$C. Elution was performed using solvent A [MeOH], solvent B [$H_2O$:MeOH (20:80) containing 0.2% ammonium acetate] and solvent C [*tert*-butyl methyl ether (MTBE)] as a linear gradient starting with 95% A and 5% B, decreasing to 80% A, 5% B and 15% C between min 2 and 10, decreasing to 30% A, 5% B and 65% C by min 30, decreasing to 25% A, 5% B and 70% C at min 40 and returning to 95% A and 5% B by min 45 (Table 2.4 A). This is a modified version of the elution gradient described by Fraser et al. (2000). An aliquot (50µL) of the sample was injected on the HPLC. The majority of carotenoids were detected at 450 nm, although other carotenoids and carotenoid precursors were monitored at 430 nm and 350 nm. The carotenoid content was determined as β-carotene equivalents/gDW of tissue. The β-carotene, and lutein were identified in the extracts by comparison of retention times and on-line spectral data with standard samples. *Trans*-β-carotene was obtained from Sigma Chemicals (St Louis, Missouri, USA). Other carotenoids were tentatively identified by comparison with reported retention times and spectral data (Fraser et al., 2000; Burns et al., 2003; Xu et al., 2006; Kammfer et al., 2010; Lee et al., 2001) and by comparison with carotenoids present in a spinach sample which was also extracted using the same procedures described (Section 2.10.1).

### 3.10.4 LCMS analysis of petal carotenoid profile

LCMS was performed by Dr Joyce at Plant & Food Research, Lincoln. The LCMS system comprised a Thermo Electron Corporation (San Jose, CA, USA) Finnigan Surveyor MS pump, Thermo Accela Open Auto sampler (PAL HTC-xt with DLW), Finnigan Surveyor PDA plus detector and a ThermaSphere TS-130 column heater (Phenomenex, Torrance, CA, USA). Each prepared extract (5 µL) was separated using a mobile phase consisting of methyl-*tert*-butyl-ether (MTBE) (A) and MeOH (B) by reverse phase chromatography (YMC30, 250 x 2.1 mm), which was maintained at 30 °C with a flow rate of 200 µL/min from time 0 to 33.5 min and follow by a flow rate of 300 µL/min from 33.5 to 39 min (Table 2.4. B). A gradient was applied: $t_{min}$/A%/B% as $t_0$/5/95, $t_{33}$/62.7/37.3, $t_{33.5\text{-}36}$/90/10, $t_{36.5\text{-}39.50}$/5/95 (Table 2.4 B)

and eluent scanned by PDA (400-700nm) and API-MS (LTQ, 2D linear ion-trap, Thermo-Finnigan, San Jose, CA, USA) with atmospheric pressure chemical ionisation (APCI) in the positive mode. Data were acquired for parent masses from m/z 350–1500 amu with fragmentation down to $MS^3$. Data were processed with the aid of Xcalibar®2.10 (Thermo Electron Corporation).

# CHAPTER THREE

# RESULTS

## 3.1 Silencing of carotenoid biosynthetic genes in *E. californica*

### 3.1.1 Cloning of carotenoid biosynthetic genes

Transcript fragments of putative carotenoid biosynthetic genes were obtained by PCR (Figure 3.1), using cDNAs derived from *E. californica* petals as templates and primers described in Table 2.1. The fragments of expected sizes were cloned into the pGEM®-T Easy vector and subsequently sequenced. The sequences were confirmed as being highly similar to carotenoid biosynthetic genes by comparison with the sequences in the NCBI GenBank using the blastn algorithm (Altschung et al 1997). To obtain longer fragments of the genes, sequences of the cloned fragments were sent to Dr Mark Fiers (Plant & Food Research) who used them to search the 454 sequence database of the transcriptome of *E. californica* petals. The 454 database search identified single hits for all carotenoid genes, except for *EcaZEP* where two small fragments were identified. However, these two fragments aligned to different regions in the *EcaZEP* transcript and so they may in fact be part of the same transcript. The sequence of each putative carotenoid biosynthetic transcript obtained from the 454 database is listed in Appendix 2.

To clone fragments of carotenoid genes for making TRV construct, PCR reaction was carried out using *E. californica* petals cDNA as the template and primers listed in Table 2.2. The PCR fragments were first inserted into pGEM®-T Easy vector for sequencing. The sequence confirm carotenogenesis gene fragments were digested from pGEM®-T Easy with restriction endonucleases *Sac*I and *Xba*I and then subcloned into the VIGS vector TRV2 as illustrated in Figure 2.1. A total of six carotenoid biosynthetic gene fragments were cloned into TRV2 and the constructs designated respectively as TRV2-*EcaPSY*, TRV2-*EcaPDS*, TRV2-*EcaZDS*, TRV2-*EcaLCYb*, TRV2-*EcaCHYb* and TRV2-*EcaZEP*.

### 3.1.2 Silencing of the carotenoid biosynthetic gene expression in *E. californica*

The *E. californica* plants used for inoculation had orange flowers. The seedlings (Figure 3.5 A) were inoculated with a 1:1 (v/v) liquid culture mixture of GV3101 agrobacteria containing TRV1 and TRV2-derived vectors. The TRV2 vector contained fragments of the carotenoid biosynthetic genes mentioned above and the control empty vector TRV2 (TRV-EV), which contained no plant insert. Plants at seven-weeks old were inoculated by both agro-infiltration and agro-drench. Agroinfiltration was done each day for the first two days. On day four, the plants were then agro-drenched on every alternative day for four days or until photobleaching was observed on leaves of the TRV-*pds* plants as the maker for VIGS.

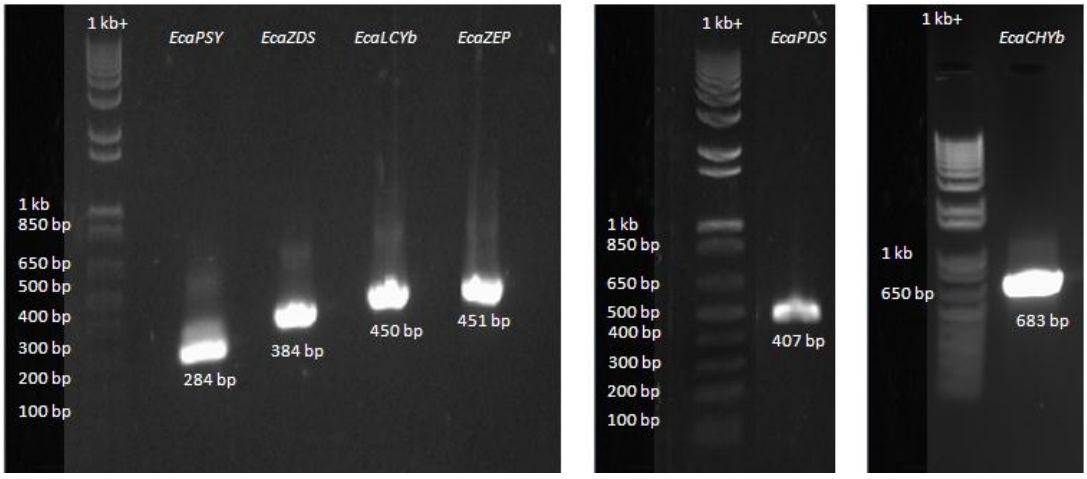



**Figure 3.1:** Carotenoid biosynthetic gene fragments as indicated, amplified by RT-PCR for insertion into the TRV2 vector. The gene-specific primers used carried restriction sites *Sac*I and *Xba*I at their 5′- and 3′-ends. The PCR template was cDNA isolated from orange wild type *E. californica* flower petals. The amplified products were separated in a 1% (w/v) TBE agarose gel. Amplicon sizes expected are labelled under each band. The expected amplicon were compared against the 1 Kb Plus DNA Ladder (Invitrogen). Products of expected size were cut from the gel for insertion into TRV2.

### 3.1.3 Confirmation of silencing in the TRV-infected lines by qRT-PCR

#### *3.1.3.1 Petal samples and RNA quality*

Alterations in flower colour caused by inoculation of the plants with the various TRV constructs were most easily observed at anthesis (pollen shed initiation) when the flower was fully open and the colour completely developed. Because of this, all petal samples for qRT-PCR were collected from *E. californica* flowers at this stage. Isolation of high quality RNA is essential for accurate gene expression profiling by qRT-PCR and the quality of the RNA isolated from the flower petals of *E. californica* was monitored using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). RNA preparations having an $OD_{260}/OD_{280}$ ratio of 1.8 to 2 and an $OD_{260}/OD_{230}$ ratio >1.8 were used. These ratios reflect low protein contamination and low polysaccharide contamination respectively (Table 3.1). The integrity of the RNA was confirmed by separating it through a 1.2% (w/v) MOPS agarose denaturing gel and observing the presence of ethidium-bromide-stained 18S and 28S rRNA bands under UV (Figure 3.2).

#### *3.1.3.2 Primer design and specificity of qRT-PCR*

Quantitative real time PCR (qRT-PCR) was used to confirm that the gene transcript targeted by VIGS was silenced. The qRT-PCR primers used for determining transcript abundance of the carotenoid biosynthetic enzymes are listed in Table 2.3 and Appendix 2. To ensure that qRT-PCR would detect only the endogenous carotenoid biosynthetic transcripts originated from the plant materials but not from the fragments cloned into the viral TRV2 vector, at least one of the primer pair used in the qRT-PCR was designed to a transcript region outside of the fragment cloned into the virus. The sequences cloned into TRV2 and the primer binding regions for each targeted transcript are both shown in Appendix 2. The specificity of each primer to its targeted gene was verified by the presence of a single, sharply defined melting curve with a narrow peak in the melt curve analysis (Figure 3.3 B) and by the detection of a single PCR product (except a minor second band was found for *EcaPSY* primers under a light saturated condition) of the qRT-PCR amplicons in a 2% (w/v) agarose gel (Figure 3.3 C). The amplification efficiencies of *ca* 2 (1.93 to 2.04) in combination with low Take Off Points (14 to 23 cycles) suggests that the PCR was efficient and that the starting amount of cDNA was in a good range for the analysis (Figure 3.3 A).

qRT-PCR was used to compare transcript abundance of the VIGS-targeted gene transcript with its abundance in wild type and TRV-EV-infected plants. qRT-PCR analysis revealed that the transcript abundance of the carotenoid biosynthetic genes in the TRV-EV flower petals was <1.5-fold different to that in wild type flowers. Therefore, transcript abundance in flowers of TRV-EV plants was used as the control in the qRT-PCR analysis. VIGS successfully and substantially reduced the transcript accumulation of all targeted genes in the petals tested. For instance, VIGS of *EcaPDS*, *EcaZDS*, *EcaLCYb*, *EcaCHYb* and *EcaZEP* reduced transcript abundance in petals 30.4-, 14-, 13.7-, 4.5-, and 4-fold respectively in comparison with petals of the TRV-EV plants (Figure 3.4). Because of both sets of replicate (from completely different run of reveres transcription and qRT-PCR) gave similar results in the final qRT-PCR, contamination effect was most likely negligible. However, for better practice of qRT-PCR for the future, non-RT control and water black should be included. Since there was only one TRV-*psy* flower available, it was not included in the qRT-PCR analysis.

**TABLE 3.1:**

Concentration and purity of RNA isolated form from *E. californica* flowers petals treated with TRV-based VIGS vectors

| **Sample[1] #** | **Sample ID** | **ng/ul** | **A260** | **A280** | **260/280** | **260/230** |
|---|---|---|---|---|---|---|
| 1 | **EV[2]** | 331.84 | 8.3 | 3.9 | 2.3 | 2.39 |
| 2 | **WT[3]** | 219.44 | 5.49 | 2.56 | 2.15 | 2.35 |
| 3 | **TRV-*pds*** | 209.22 | 5.23 | 2.54 | 2.02 | 2.47 |
| 4 | **TRV-*zds*** | 300.81 | 7.52 | 3.5 | 2.15 | 2.37 |
| *5* | ***TRV-lcyb*** | 406.15 | 10.15 | 4.76 | 2.13 | 2.33 |
| 6 | **TRV-*chyb*** | 168.13 | 4.2 | 1.93 | 2.18 | 2.32 |
| 7 | **TRV-*zep*** | 192.9 | 4.82 | 2.24 | 2.15 | 2.35 |
| 8 | **EV** | 632.48 | 15.81 | 7.44 | 2.13 | 2.09 |
| 9 | **TRV-*pds*** | 456.06 | 11.40 | 5.48 | 2.08 | 2.32 |
| 10 | **TRV-*zds*** | 397.94 | 9.95 | 4.72 | 2.11 | 2.37 |
| 11 | **TRV-*lcyb*** | 343.12 | 8.58 | 4.03 | 2.13 | 2.34 |
| 12 | **TRV-*chyb*** | 328.66 | 8.216 | 3.83 | 2.15 | 2.22 |
| 13 | **TRV-*zep*** | 440.12 | 11 | 5.15 | 2.14 | 2.33 |

[1]Samples 1-7 (replicate 1), samples 8-13 (replicate 2)

[2]Empty vector control

[3]Wild type

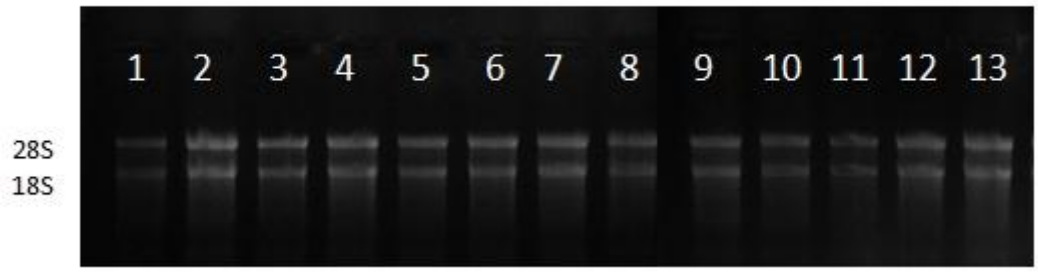



**Figure 3.2:** Integrity of the RNA isolated from petals of TRV-infected plants as shown by ethidium bromide staining. RNA was resolved in a 1.2 % MOPS denaturing agarose gel and visualized under UV. Each lane (1-13) corresponds to one RNA sample as defined in Table 3.1, and lane contains 5 μL of the RNA preparation. The 18S and 28S rRNAs are indicated.

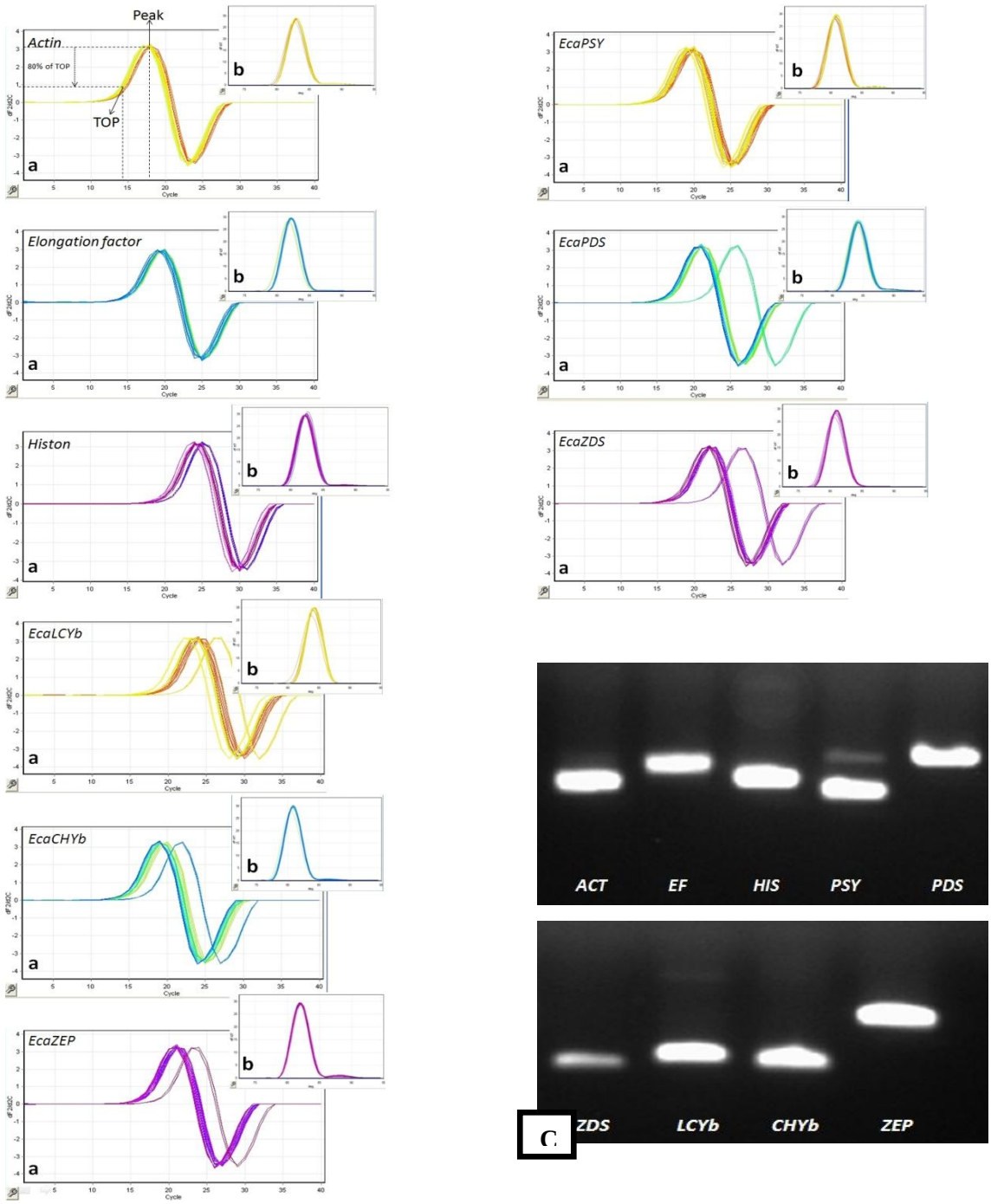



**Figure 3.3:** Quality control for the qRT-PCR data. **a**, Second derivative plot of raw fluorescence data for each primer set. Data for each of the 5 silenced lines, wild type and TRV-EV lines are shown for each primer set as indicated. The peaks indicate the maximum rate of exponential amplification. The take off point (TOP), which is equivalent to the threshold cycle ($C_t$) in other analysis methods, is defined as the cycle number where amplification is 80% below its peak value. **b**, Melt curve analysis of each primer set, where a sharply defined curve with a narrow peak is suggestive of a single amplified product. **c**, Amplification products of the qRT-PCR were resolved in a 2% agarose gel.

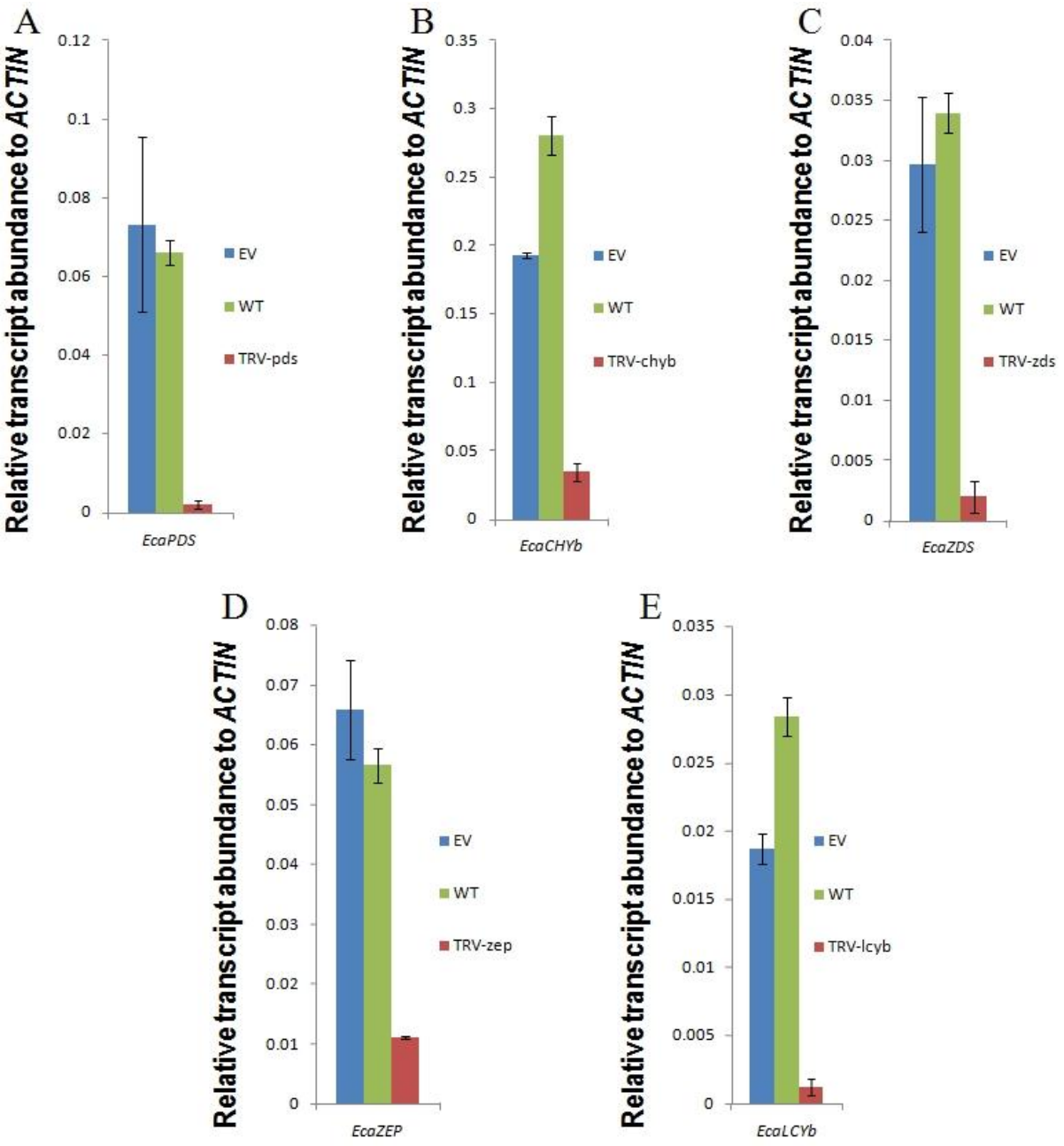



**Figure 3.4:** Efficacy of VIGS in silencing targeted gene transcripts in petals.
Relative transcript abundance of the carotenoid biosynthetic gene in petals of the VIGS-targeted plant versus wild type and TRV-EV infected plants. **A**, *EcaPDS*. **B**, *EcaCHYb*. **C**, *EcaZDS*. **D**, *EcaZEP* **E**, *EcaLCYb*. Transcript abundance was normalized to the abundance of *ACTIN*. Values are means ± STDEV of biological replicates (*n*=2), with each biological replicate consisting of pooled petal samples from 4 to 5 plants. The primers used in the qRT-PCR are listed in Table 2.3.

### 3.1.4 Effect of the silenced carotenoid biosynthetic genes on plant development, leaf and flower colouration

Infection of the wild type *E. californica* seedlings with TRV2-EV (used as the experimental control) and TRV1 did not retard plant growth or cause visual alteration in plant pigmentation of leaves or flowers when the plants were observed firstly under white light (Figure 3.5C and 3.6B) and then UV light (data not shown). In contrast, plants infected with TRV containing fragments of plant carotenoid biosynthetic genes displayed a number of leaf and flower phenotypes and were also slower in growth, which was dependent upon the biosynthetic gene silenced (Figure 3.5 and Figure 3.6).

Down-regulating transcripts in the early steps of the carotenoid biosynthetic pathway (*EcaPSY*, *EcaPDS* and *EcaZDS*) caused photobleaching of leaves (Figure 3.5D-F). All seedlings infected with TRV2-*EcaPDS* and TRV2-*EcaZDS* showed extensive leaf photobleaching at 10 days post inoculation (dpi), illustrating both the efficacy of the inoculation procedure and the VIGS approach. The plants inoculated with TRV-*psy* also exhibited photobleaching, but to a lesser degree compare with the TRV-*pds* and TRV-*zds* inoculated plants. One hundred percent of the plants inoculated with TRV2-*EcaPDS* and TRV2-*EcaZDS* showed strong photobleaching, whereas only 33% of the 18 TRV-*psy* plants showed some degree of photobleaching. Most of the photobleaching was partial, occurring in the middle of the new leaves around the vascular structures. Only two TRV-*psy* plants showed extensive photobleaching. The TRV-*pds*- and TRV-*zds* plants also grew noticeably slower than the controls. In contrast to silencing genes in earlier part of the pathway, silencing of the gene expression involved in the later steps (i.e., *EcaLCYb*, *EcaCHYb* and *EcaZEP)* of the carotenoid pathway did not show any obvious photobleaching in leaves. There was also no clear change in pigmentation when the plants were observed under UV light.

In the flower, silencing each of the five genes *EcaPSY*, *EcaPDS*, *EcaZDS*, *EcaCHYb* and *EcaZEP* in the carotenoid biosynthetic pathway led to distinct changes in petal pigmentation (Figure 3.6D-H). Approximately 70% of flowers showed an extensive silencing colour phenotype, which is defined as the flower having retained its orange colour in less than 20% of the total petal area. However, the flowers of the TRV-*lcyb* plants did not show any visual alteration in flower colour (Figure 3.6F). Flowers of TRV-*psy*, TRV-*pds* and TRV-*zds* plants displayed an albino phenotype. The loss of colour was more pronounced in the TRV-*pds* and

TRV-*zds* flowers when compare with in the TRV-*psy* flowers. The TRV-*pds* and TRV-*zds* flowers were often white in most of the petal with a small amount of pigment present around the edge; it was light orange for TRV-*pds* and light yellow for TRV-*zds* flowers. Due to a technical problem, only one true TRV-*psy* flower was obtained before the flowering season was over. This flower showed differential loss of colour in a sector-like pattern. The flower of TRV-*chyb* was light orange in colour (Figure 3.6G) and much fainter than that of the TRV-EV (Figure 3.6B). The TRV-*zep* flowers were orange-yellow and occasionally had dark-orange stripes(Figure 3.6H).

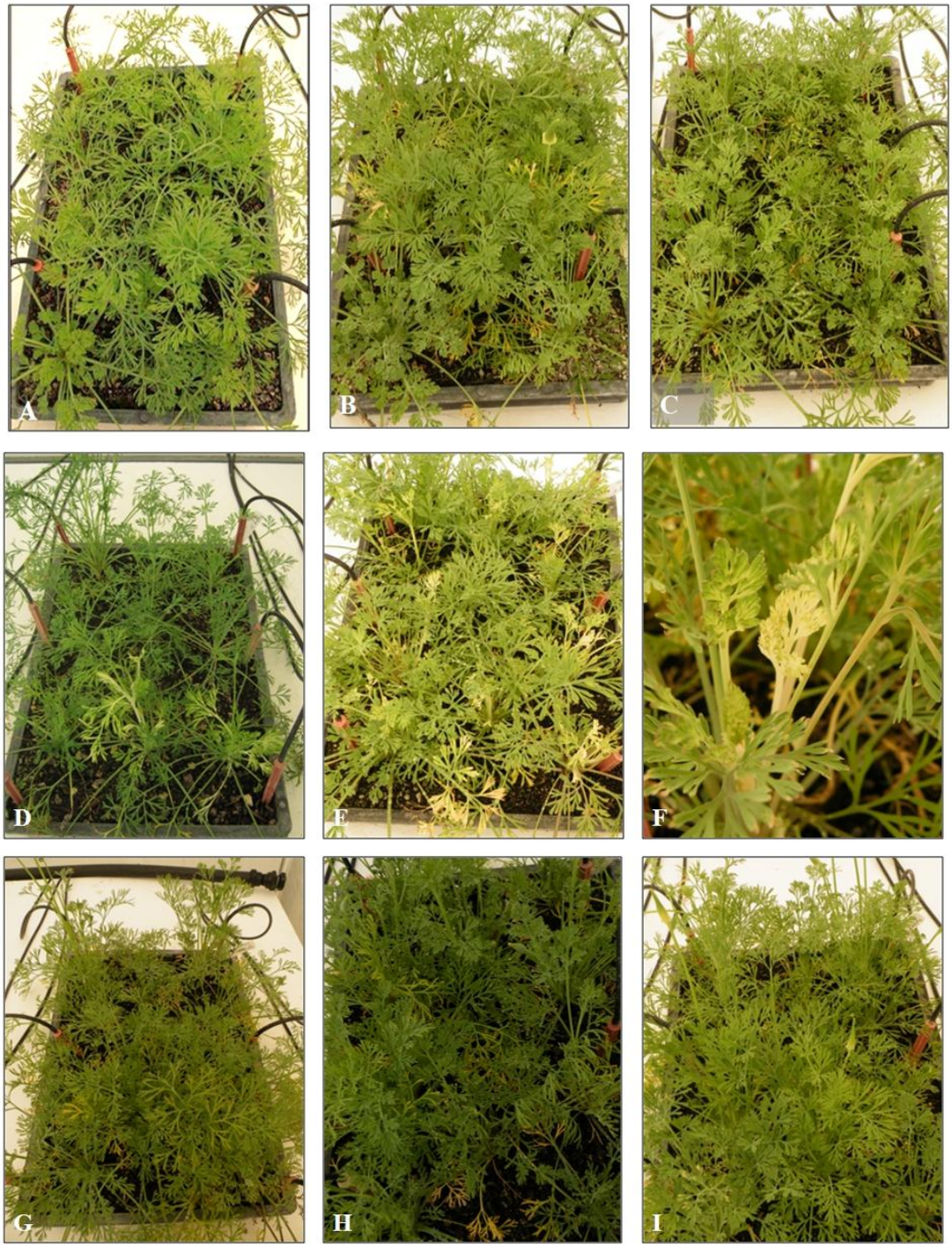



**Figure 3.5:** Leaf phenotypes of the TRV-infected plants. **A**, Seven-week-old wild type *E. californica* seedings before inoculation. **B-H,** Leaves of wild type and TRV-infected plants 13 days post-inoculation. **B**, Wild type. **C**, TRV-EV. **D**, TRV-*psy*. **E**, TRV-*pds*. **F**, TRV-*zds*. **G**, TRV-*lcyb*. **H**, TRV-*chyb*. **I**, TRV-*zep*. Plants were grow in Viral House unit which was maintained by air conditioning units and were set to 22 °C.


**Figure 3.6:** Flower colour phenotypes of the TRV-infected lines. **A**, Wild type; **B**, Empty vector (EV) control; **C**, TRV-*psy*; **D**, TRV-*pds*; **E**, TRV-*zds*; **F**, TRV-*lcyb*; **G**, TRV-*chyb*; **H**, TRV-*zep*. Petals were collected at anthesis, imaged and follow by frozen in liquid nitrogen and stored at -80ºC for further analyses.

## 3.2 Changes in carotenoid metabolites caused by gene silencing

Down-regulating gene expression at particular steps of the carotenoid biosynthetic pathway can affect flow of metabolites through the pathway. To study how the whole carotenoid metabolite profile was affected, spectrophotometry and HPLC were used. These techniques enabled the determination of total carotenoid content, and the change in relative abundance of the different carotenoid types. The identity of carotenoid metabolites was later confirmed by LC-MS. The carotenoid profile change in *E. californica* petals could then be analysed along side with the gene silencing data.

### 3.2.1 Total carotenoid content of petals

Spectrophotometry and HPLC measurements produced similar results in terms of total carotenoid content in the petals when a methanolic/acetone solvent was used to extract the carotenoids (Figure 3.7). The total carotenoid content in the petals of the TRV-EV-infected plants was not significantly different to that of wild type. By contrast, the total carotenoid content in the petals of VIGS plants varied considerably depending upon the biosynthetic gene silenced. Silencing of *EcaPDS*, *EcaZDS*, *EcaCHYb* and *EcaZEP*, but not *EcaLCYb* genes caused a significant reduction (≥ 3-fold) in the total carotenoid content of the flower petals. Total carotenoid content was reduced 16-fold in TRV-*pds* petals, 11-fold in TRV-*chyb* petals, 4-fold in TRV-*zds* petals and 3-fold in TRV-*zep* petals when compared with the TRV-EV petals.

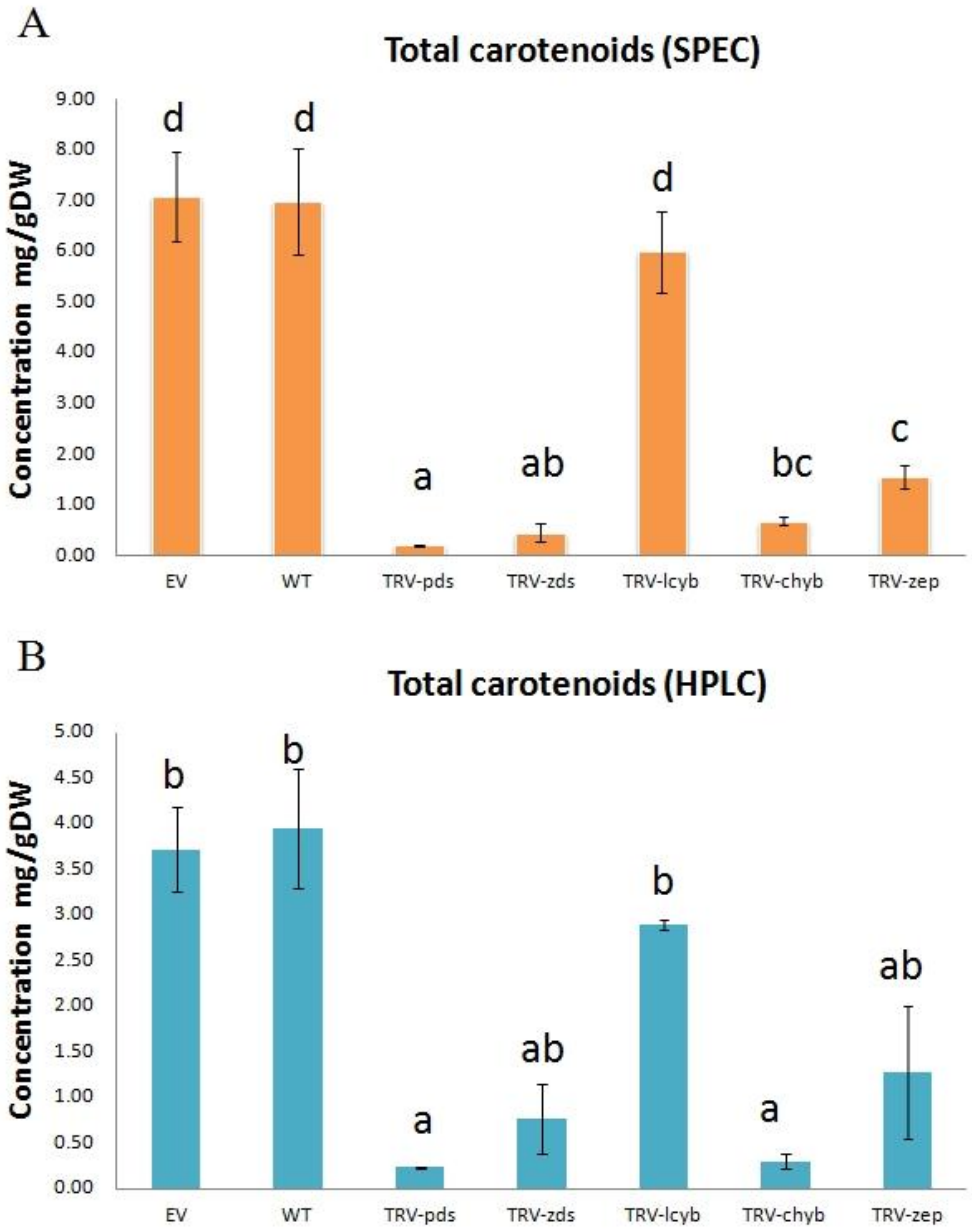



**Figure 3.7:** Total carotenoid content of petals of wild type plants and TRV-infected plants as indicated. **A**, Spectrophotometric determination. Values are mean ± STDEV (*n*=2). **B**, HPLC determination. Values are mean ± STDEV (*n*=2). The different letters represent significant differences as determined by Tukey's 95% confidence intervals.

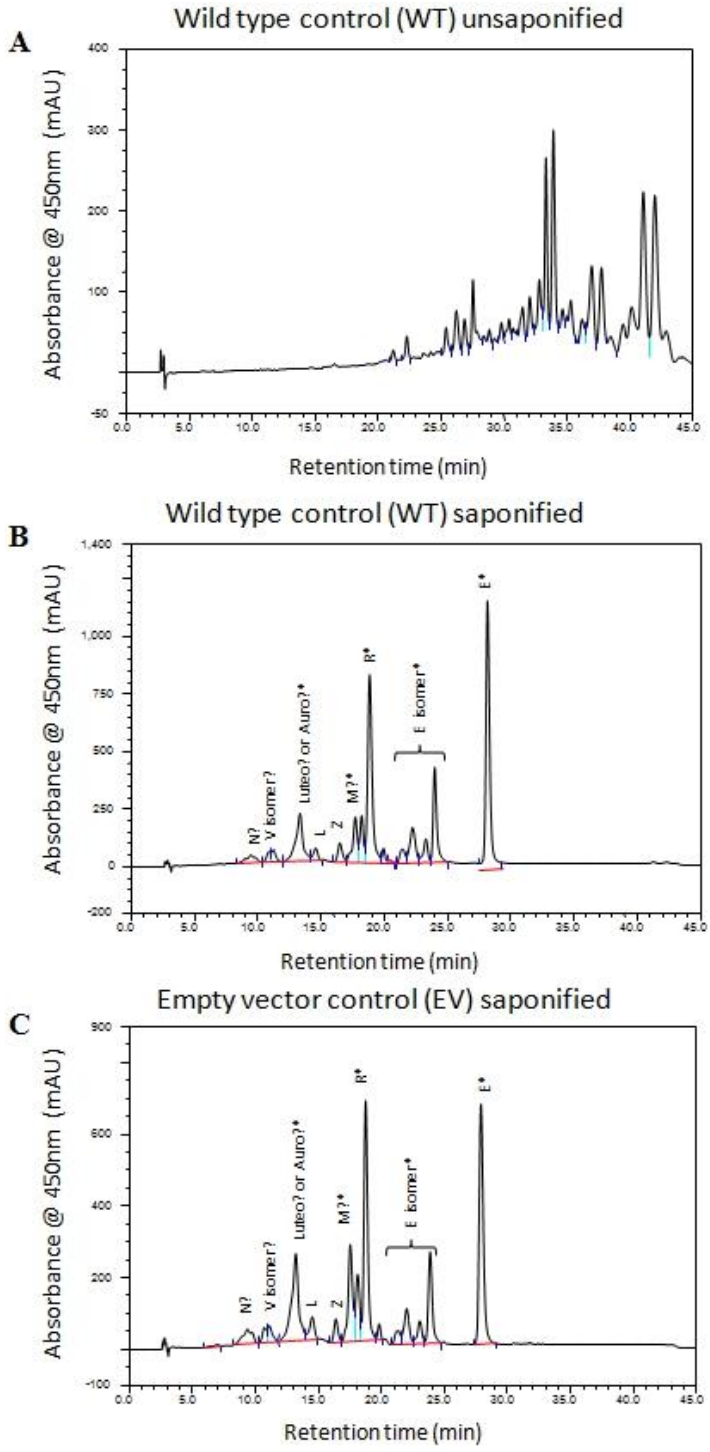
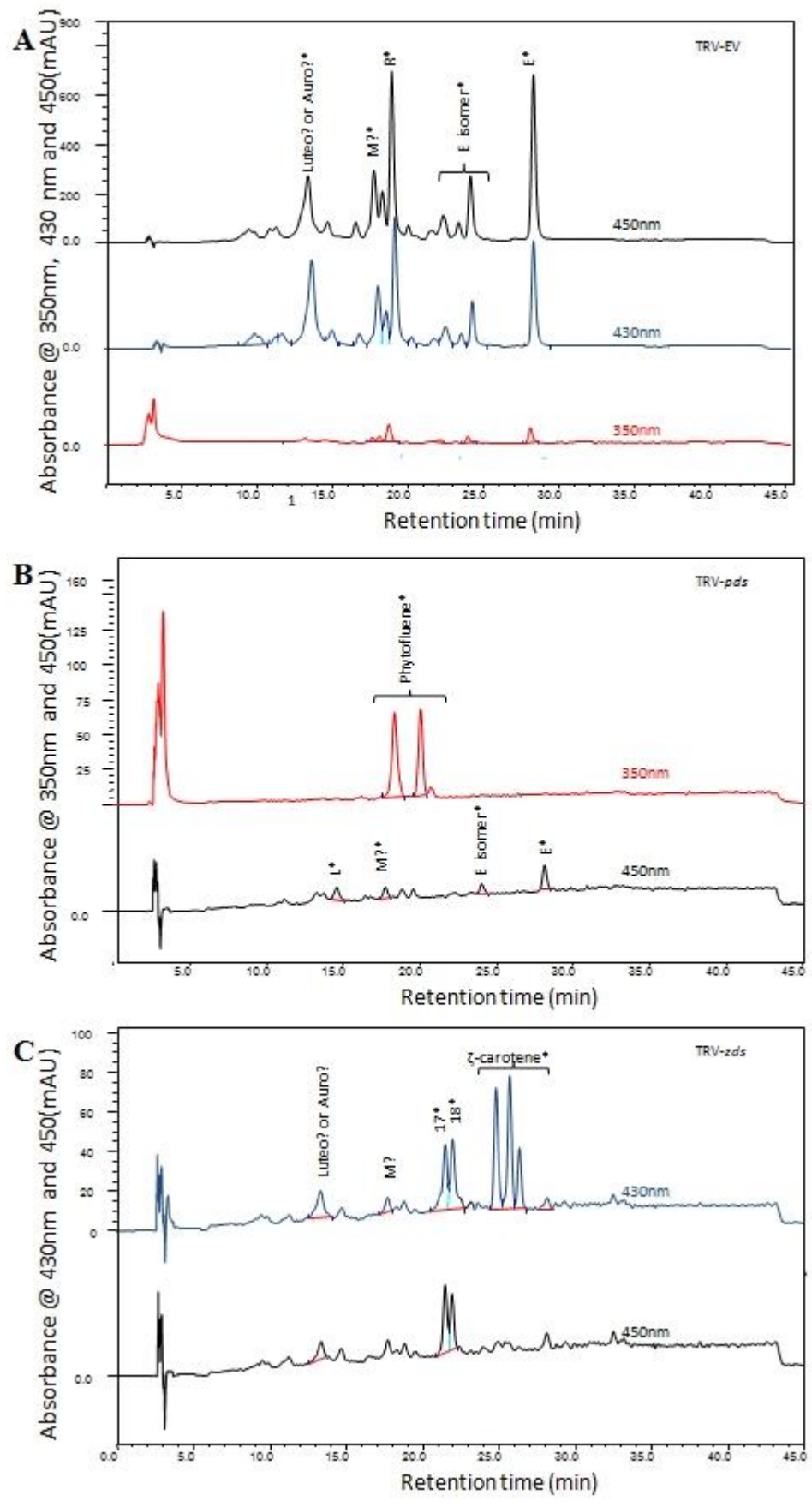



**Figure 3.8:** Representative HPLC chromatograms of methanolic/acetone extracts from orange flower petals of *E. californica* wild type or TRV empty vector control plants as indicated. **A**, unsaponified sample of wild type **B,** saponified wild type. **C,** saponifed TRV-EV. Absorbance was monitored at 450 nm. The four major types of carotenoid end products, which are identical between wild type and TRV-EV petals are indicated by *. N (Neoxanthin), V isomer (Violoxanthin isomer), Lxanthin (Luteoxanthin), Axanthin (Auroxanthin), L (Lutein), Z (Zeaxanthin), M (Mutatoxanthin), R (*Retro*-carotene-triol x *E.californica*), E isomer (Eschscholtzxanthin isomer) and E (Eschscholtzxanthin). Of these any tentatively identified peaks are indicated by ?.


A
TRV-EV
Lutein? or Auro?*
M?*
R*
E isomer*
E*
450nm
430nm
350nm
Absorbance @ 350nm, 430 nm and 450(mAU)
Retention time (min)
B
TRV-pds
Phytofluene*
350nm
L*
M?*
E isomer*
E*
450nm
Absorbance @ 350nm and 450(mAU)
Retention time (min)
C
TRV-zds
Lutein? or Auro?
M?
17*
18*
ζ-carotene*
430nm
450nm
Absorbance @ 430nm and 450(mAU)
Retention time (min)

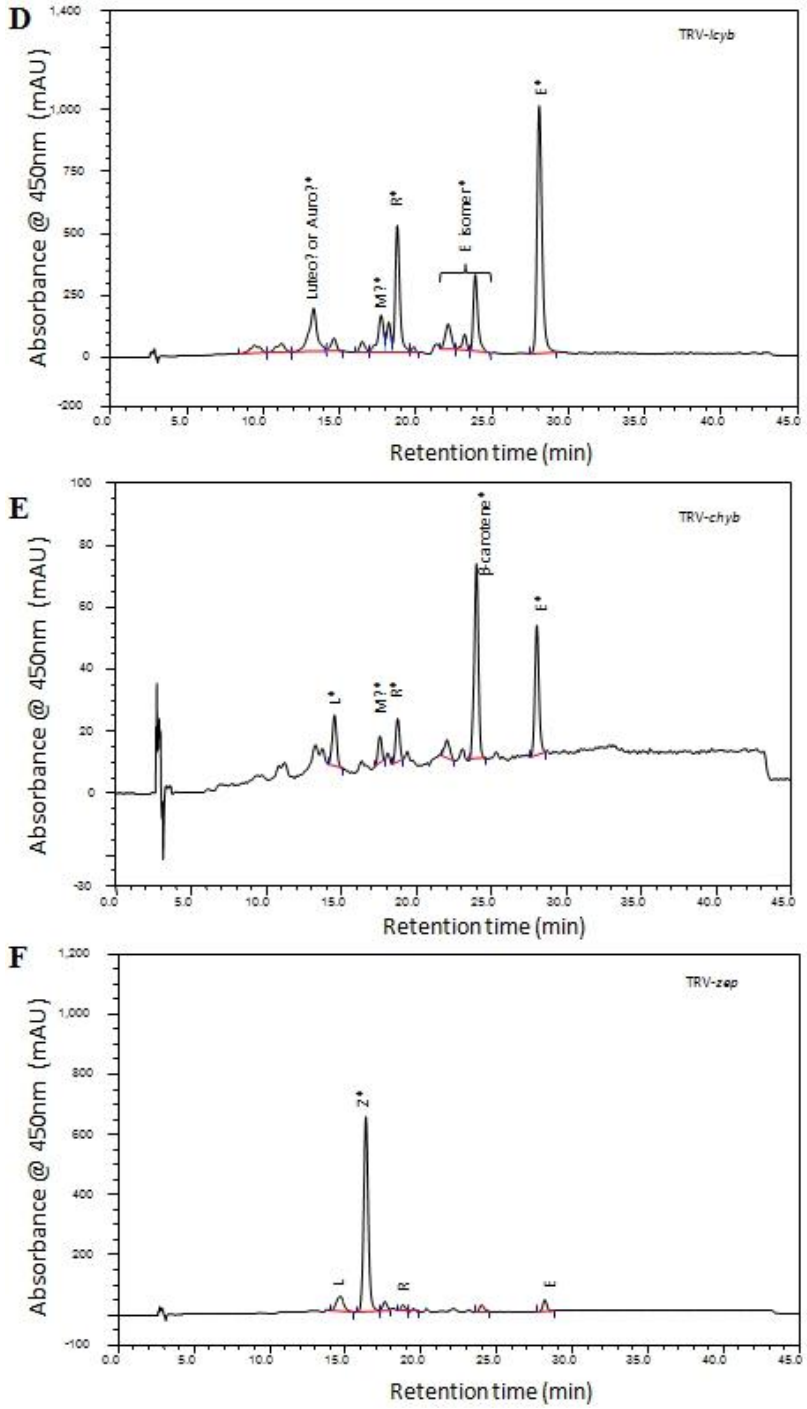



**Figure 3.9:** Representative HPLC chromatograms of saponified methanolic/acetone extracts from flower petals of *E. californica* plants infected with TRV as indicated. **A**, TRV-EV. **B**, TRV-*pds*. **C**, TRV-*zds*. **D**, TRV-*lcyb*. **E**, TRV-*chyb*. **F**, TRV-*zep*. Absorbance was monitored at 450 nm for all lines. Absorbance was also monitored at 350 nm for TRV-*pds* and 430 nm for TRV-*zds*. Major types of carotenoid end products are indicated by *. Carotenoids underlying the chromatogram peaks are indicated where known. The unknown peaks are numbered 17 and 18 (Table 3.2), tentatively identified peaks are indicated by ?. Each of the multiple plots in the graph has same scale.

### 3.2.2 Identification and quantification of the carotenoid metabolites in petal tissue

VIGS of carotenoid biosynthetic genes caused changes in the carotenoid pathway intermediates and end products in the petal tissues. Representative HPLC traces of the carotenoid metabolites in petal extracts from plants with different biosynthetic genes silenced are shown as Figures 3.8 and 3.9. Retention times between 20 min to 45 min were obtained for the analysis of the natural forms of carotenoids in the petal extracts, where a longer retention time and less clear separation patterns indicated the presence of esterified carotenoids (Figure 3.8A). Because esterified carotenoids complicate identification of the parent carotenoid by altering retention times, saponification was performed on the carotenoid extract to cleave off the ester groups. The saponified extracts were then subjected to HPLC to identify the parent carotenoids (Figure 3.8B-C). Each peak corresponds to a particular carotenoid species and the area under the peak represents the amount of that particular metabolite. The identity of corresponding peak was determined by comparison of retention times to previously reported spectral data (Fraser et al., 2000; Burns et al., 2003; Xu et al., 2006; Kammfer et al., 2010; Lee et al., 2001) and the sequence of peak emergence. β-carotene and lutein were identified in the extracts by comparison of retention times and on-line spectral data with standard samples. The spectrum readings of the carotenoid metabolites in this study are listed in Table 3.2.

To further verify the identities of the peaks detected with the 450 nm wavelength, LC-MS was used to analyse the saponified samples of wild type flower petals in an attempt to cover all the peaks found in the petals. The details of the mass fragmentation of the other peaks are shown in Table 3.2.

**TABLE 3.2:**

Summary of the spectral properties and identification of carotenoids extracted from wild type and TRV-infected plants using LCMS

| Peak | Retention time (min) | Spectral Maxima | Parent $[M+H]^+$ | ms2 | Identity |
|---|---|---|---|---|---|
| Wild type Saponified detected at 450nm | | | | | |
| 1 | 9.455 | 410/436/465 | - | - | [1]Neoxanthin? |
| *2* | 10.872 | 422/445/468 | - | - | Violoxanthin isomer? |
| 3 | 11.247 | 422/445/468 | - | - | Violoxanthin isomer? |
| 4 | 13.323 | 411/434/463 | 601 | 583 | Luteoxanthin or Auroxanthin? |
| 5 | 14.627 | 420/443/471 | - | - | Lutein |
| 6 | 16.495 | 415/450/476 | - | - | Zeaxanthin |
| 7 | 17.718 | 415/441/467 | - | - | Mutatoxanthin? |
| 8 | 18.253 | 425/454/481 | - | - | Unknown |
| 9 | 18.850 | 434/458/487 | 585 | 567 | *retro*-carotene-triol x *E.californica* |
| 10 | 19.970 | 420/450/476 | - | - | Unknown |
| 11 | 20.515 | 420/450/476 | - | - | Unknown |
| 12 | 21.432 | 438/459/488 | 566 | 549,531 | Eschscholtzxanthin isomer |
| 13 | 22.213 | 440/463/492 | 566 | 549, 531 | Eschscholtzxanthin isomer |
| 14 | 23.265 | 443/465/494 | 566 | 549,531 | Eschscholtzxanthin isomer |
| 15 | 24.013 | 444/467/497 | 566 | 549, 531 | Eschscholtzxanthin isomer |
| 16 | 28.140 | 446/470/501 | 566 | 549,531 | Eschscholtzxanthin |

| | | | | | |
|---|---|---|---|---|---|
| ZDS Saponified peaks detected at 430nm | | | | | |
| 4 | 13.355 | 411/434/463 | 601 | 583 | Luteoxanthin or auroxanthin? |
| 5 | 14.689 | 420/443/471 | - | - | Lutein |
| 7 | 17.760 | 415/441/467 | - | - | Mutatoxanthin? |
| 9 | 18.880 | 434/458/487 | 585 | 567 | *retro*-carotene-triol x E.californica |
| 17 | 21.557 | 406/427/452 | | | Unknown |
| 18 | 22.002 | 406/427/452 | | | Unknown |
| 12 | 24.022 | 448/467/496 | - | - | Eschscholtzxanthin isomer |
| 19 | 24.909 | 380/400/425 | 541 | 446,403 | ζ – carotene 1 |
| 20 | 25.769 | 380/400/425 | 541 | 446,403 | ζ – carotene 2 |
| 21 | 26.390 | 380/400/425 | | | ζ – carotene 3 |
| 16 | 28.199 | 446/470/501 | 566 | 549,531 | Eschscholtzxanthin |

[1] Tentatively identified peaks were indicated by ?.

HPLC traces were recorded at 450 nm, 430 nm and 350 nm wavelengths because these are the wavelengths that carotenoid metabolites are known to have maximal absorption. Most of the major carotenoid metabolites absorb at 450 nm and so chromatograms for this wavelength were recorded for all VIGS-infected plants (Figures 3.9). Phytofluene and phytoene have spectral maxima at 350 nm and poorly absorb at 450 nm, so additional chromatograms at 350 nm are shown for TRV-*pds* (Figure 3.9B). Similarly, as the absorption peak for ζ-carotene is 430 nm this wavelength was monitored for TRV-*zds* petal extracts (Figure 3.9C).

Wild type and TRV-EV petal extracts monitored at 450 nm had similar carotenoid profiles based on their retention times and heights of their recorded peaks (Figure 3.8B-C). The four major end products of the carotenoid biosynthetic pathway in orange flower petals of *E. californica* are luteoxanthin/auroxanthin, mutatoxanthin, retro-carotene-triol and eschscholtzxanthin. *Retro*-carotene-triol and eschscholtzxanthin are the most abundant carotenoids, which each of them accounting for over 20% and 40% of the total carotenoid content respectively. Luteoxanthin/auroxanthin was 14.11% of the total content, while mutatoxanthin took up 9.92% of the total content. Other minor end products were also identified, such as neoxanthin, violaxanthin, lutein, zeaxanthin and three unidentified carotenoids, but these only constituted a small proportion of the total carotenoid profile in the orange *E. californica* flower petals.

VIGS of the carotenoid biosynthetic genes (except *EcaLCYb*) caused substantial changes in the types and relative proportions of the carotenoids in the *E. californica* petal extract (Figure 3.9). TRV-*pds* petal extracts had dramatically reduced amounts of end products in the pathway as determined by measurement at 450 nm (Figure 3.9B). Measurement at 350 nm revealed two distinct peaks characteristic of phytofluene, the substrate of PDS. These peaks were absent in TRV-EV petal extracts (Figure 3.9B). The TRV-*zds* petal extracts also had a substantial reduction in all end products as well as the appearance of two new unknown peaks (unknown 17 and 18) at 450 nm (Figure 3.9C). Measurement at 430 nm revealed the presence in TRV-*zds* petal extracts of ζ-carotene (57%), the two unknown peaks (17, 18), a slightly reduced percentage of luteoxanthin/auroxanthin, mutatoxanthin and a complete disappearance of *retro*-carotene-triol and eschscholtzxanthin (Figure 3.9C). The TRV-*chyb* petal extracts showed a substantial reduction in percentage of all major end products except eschscholtzxanthin. The percentage of lutein increased from 2% to 11.6% of the total carotenoid content of the tissue, and β-carotene, which was not detected in the TRV-EV petal

extracts, became the major carotenoid (43.1%) (Figure 3.9G). TRV-*zep* petal extracts had a substantial reduction in all major end products except zeaxanthin whose percentage increased from 2% to 78% of the total carotenoids in the tissue (Figure 3.9F). On top of this increase in percentage, the zeaxanthin content increased from 0.14 mg/gDW in the EV control to 1.2 mg/gDW in the TRV-*zep* petal extract.

## 3.3 Gene expression profiling

### 3.3.1 Transcript abundance of the carotenoid biosynthetic genes in flower petals of control plants at anthesis

Transcript abundance of *EcaPSY* and *EcaCHYb* was much higher than the other gene transcripts in petals of flowers at anthesis in both wild type and TRV-EV infected plants. Their transcript abundance was more than 3-fold higher than the abundance of the other transcripts. The *EcaPDS* and *EcaZEP* transcripts were the next most highly abundant being *ca* 2-fold more than the *EcaZDS* and *EcaLCYb* transcripts (Figure 3.10).

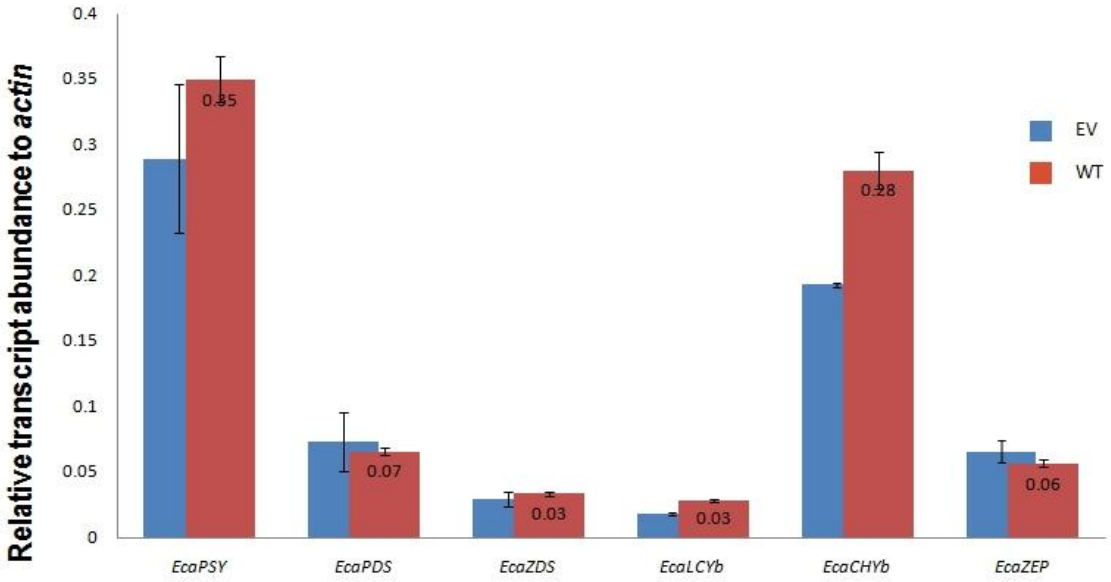



**Figure 3.10:** Transcript abundance of carotenoid biosynthetic genes in petals of wild type and TRV-EV plants determined using qRT-PCR. The petals were harvested at anthesis, and transcript abundance was normalized to the averaged abundance of *ACTIN*. Values are mean ± STDEV of biological replicates (*n*=2), with each biological replicate consisting of pooled petal samples from 4 to 5 plants.

**TABLE 3.3:**

Transcript abundance of carotenoid biosynthetic genes in petals of TRV-infected plants. Petals were harvested at anthesis. Transcript abundance was normalized to the averaged abundance of *ACTIN* and *ELOGATION FACTOR*. Values are mean ± STDEV of biological replicates (*n*=2), with each biological replicate consisting of pooled petal samples from 4 to 5 plants.

| Genes / silenced plants | *EcaPSY* | *EcaPDS* | *EcaZDS* | *EcaLCYb* | *EcaCHYb* | *EcaZEP* |
|---|---|---|---|---|---|---|
| TRV-EV | 1 | 1 | 1 | 1 | 1 | 1 |
| Wild type | 0.98 | 1 | 1 | 0.82 | 1.78 | 0.97 |
| TRV-*pds* | 1.23±0.19 | 0.03 | 0.99±0.09 | 0.92±0.17 | 1.65±0.42 | 1.06±0.02 |
| TRV-*zds* | 1.13±0.09 | 0.74±0.1 | 0.03±0.01 | 1.05±0.1 | 1.11±0.44 | 0.67±0.05 |
| TRV-*lcyb* | 0.85±0.09 | 1.06±0.03 | 0.9±0.02 | 0.11±0.05 | 1.6±0.03 | 0.96±0.18 |
| TRV-*chyb* | 1.68±0.15 | 1.17±0.06 | 1.34±0.01 | 2.19±0.12 | 0.21±0.03 | 1.14±0.18 |
| TRV-*zep* | 0.71±0.11 | 0.9±0.10 | 1.07±0.22 | 1.02±0.03 | 1.24±0.21 | 0.14±0.01 |

### 3.3.2 Transcript abundance of all carotenoid biosynthetic genes in the VIGS plants

To determine whether silencing a particular carotenoid biosynthetic gene affected transcript abundance of other genes in the pathway, the transcript abundance of *EcaPSY*, *EcaPDS*, *EcaZDS*, *EcaLCYb*, *EcaCHYb* and *EcaZEP* was determined by qRT-PCR in each of the VIGS-infected plants. Silencing of the individual genes of the carotenoid biosynthetic pathway did not lead to substantial changes in transcript abundance of the other genes in the pathway (Table 3.3). The exception was *EcaLCYb*, which showed a 2.19-fold increase in the TRV-*chyb* silenced plant, No transcript showed more than a 2-fold increase in abundance in any other of the VIGS-infected plants.

# CHAPTER FOUR

# DISCUSSION AND SUMMARY

This thesis focused on a central question: Do carotenoid metabolites in chromoplasts of *E. californica* petals feedback to regulate carotenogenesis-related transcription? To answer this, gene fragments of most of the carotenoid biosynthetic genes from *E. californica* were cloned and used in a VIGS-based approach to transiently silence their transcript accumulation in flower petals of *E. californica.* HPLC and LC-MS analysis of petal extracts clearly showed that individually silencing carotenogenesis genes in flower petals increased the concentration of specific up-stream metabolites and decreased the down-stream carotenoids to various extents depending on the gene targeted. The total carotenoid content of the petals was substantially decreased in all of the VIGS petals. Despite the substantial changes in carotenoid content and metabolite profiles, the transcript abundance of other non-targeted endogenous genes in the pathway was not affected, apart from a slight increase in transcript abundance of *EcaLCYb* in the TRV-*chyb* petals. This suggests first, that in *E. californica* flower petals transcriptional abundance of the genes in carotenogenesis is not dramatically affected by targeted down-regulation of a single pathway gene, and secondly, that carotenogenesis transcript abundance does not appear to be feedback regulated *via* the increased accumulation of intermediary carotenoid metabolites in petals.

## 4.1 VIGS is an effective tool for studying transcriptional regulation of carotenogenesis in *E. californica* flowers

The efficacy of VIGS can vary widely depending on the interaction between the viral vector and its host. Sometimes, plants from different cultivars of the same species respond differently to the same VIGS system. However, despite the unavoidable variation in *E. californica* plant material due to it being an outcrossing species, extensive and reproducible silencing was obtained by repeating the agro-infiltration and agro-drenching over a number of days. TRV-*pds* and TRV-*zds* plants displayed widespread photobleaching of leaves, an evenly spread of colour loss in petals and greatly reduced transcript abundance of the targeted genes in the petals. This confirmed VIGS as an effective tool for gene silencing in *E. californica* flowers.

TRV has also been successfully used to silence genes in flowers of other species. For example, TRV was used to effectively silence *PDS* in the gerbera cultivar Grizzly. The silencing led to photobleaching in leaves and the presence of albino flowers, although vacuum-infiltration was required for these phenotypes to occur (Deng et al. 2012). In *Mirabilis jalapa* plants, TRV-*pds* inoculation alone failed to cause photobleaching, but it was achieved when *PDS* was co-silenced with the antiviral protein MAP (Singh et al. 2012).

Silencing the expression of essential genes such as *PDS* and *ZDS* can cause premature death of a plant. For example, *Arabidopsis* (*pds3*) and sunflower (*non dormant-1*) seedlings defective in *PDS* or *ZDS* expression showed severe photobleaching and could not develop past the seedling stage (Conti et al. 2004; Qin et al. 2007). In contrast, the TRV-*pds* and TRV-*zds* plants of *E. californica* in the present study were still able to develop to flowering, providing a valuable system for studying the functions of essential genes and those involved in secondary metabolism.

Although wild type viruses may, depending on host and environment, cause loss of petal carotenoid content leading to the phenomenon termed 'colour break' (Hunter et al. 2011), this did not appear to be the case for TRV and *E. californica*. For instance, and in common with Wege et al. (2007) *E. californica* plants infected with a mixture of TRV1 with TRV2 empty vector did not show more noticeable changes in growth rate, leaf or floral morphology and importantly floral pigmentation patterns when compared with untreated wild type controls. The TRV empty vector has by contrast been shown to cause developmental lesions in tomato plants, which confounds their analysis (Wu et al. 2011). Wu et al. found that lesions were minimised if a non plant gene fragment such as GUS was inserted into the TRV vector and they proposed that this chimeric TRV2 was therefore a better control than the empty vector. In some instances, the dramatic effect of viruses on plant morphology has been associated with the virus interfering with plant siRNA and miRNA production which is essential for normal plant development (Bouche et al. 2006). Presumably because of the lack of effect on plant morphology observed in this study, TRV did not overload the siRNA and miRNA production pathway in *E. californica*.

Some carotenogenesis genes appear to be members of small gene families. For example, multiple *PSY* genes have been reported in many species of *Poaceae*, as well as in the *Brassiceae* and tomato (Bartley & Scolnik 1993; Fraser et al. 1999; Li et al. 2008; Cardenas et

al. 2012). However, where this has been found to occur the individual members have been shown to localise to different tissues in the plants. This may explain why I only found evidence for one gene member in the petal tissues of *E.californica* when the 454 transcript sequences (made to pooled green and orange petal tissue) were screened. However, although I cannot unequivically rule out that other members are not present, the dramatic effect of silencing the genes tested on the carotenoid profile of the petal tissue would be consistent with this.

Unexpectedly, the photobleaching of *PSY* silenced leaves of *E.californica* was not as extensive as expected when compared with that of TRV-*pds* and TRV-*zds* plants. Given the high efficacy of the experimental system, this may be accounted for by two possibilities; first, the PSY gene in leaf tissue may be different to that of the one in petal tissue, and secondly, the high level of *PSY* gene expression. Southern blotting may be carried out to verify the number of *EcaPSY* gene. With respect to the second possibility, transcript abundance of *PSY* in wild type flower petals was more than 10-fold higher than the other carotenoid genes as judged by qRT-PCR (Figure 3.10). Because VIGS was found to reduce transcript abundance of each targeted gene by a similar proportion (Figure 3.10, Table 3.3), transcript abundance of *PSY* after silencing, and therefore PSY protein abundance might still have been high enough in the silenced plant to provide sufficient flux into the carotenoid biosynthetic pathway.

The leaves of TRV-*lcyb*, TRV-*chyb* and TRV-*zep* plants did not show any visible photobleaching, and this is consistent with phenotypes observed in the flowers of these plants. Judging by the HPLC analysis of the *CHYb*–silenced petals, the carotenoid pathway has been almost blocked at β-carotene. If the same situation is found in the leaves of TRV-*chyb* plants, this does not agree with the essential roles of xanthophylls (lutein, zeaxanthin etc., where CHYb is required for their production) for photosynthesis and photoprotection (Polivka & Frank 2010). In the case of *ZEP*-silenced petals, the only carotenoid that was of significant abundance was zeaxanthin, suggesting that even a single carotenoid present in the leaves is enough to prevent photobleaching. The healthy young leaves in the plants with silenced expression of these three genes also indicate that carotenoid forms might be highly replaceable in the chloroplast to maintain the integrity of the photosynthetic systems. On the other hand, carotenoid biosynthetic pathways may be differently regulated in photosynthetic tissues and non-photosynthetic tissues, as demonstrated by the transient expression of maize PSY1 in green and etiolated protoplasts which caused different effects to the plastids of those

isolated protoplasts (Shumskaya et al., 2012). However, because apart from visual observations no further analysis was carried out on leaves, the regulation and roles of carotenoids in the leaf tissue can only at this point be speculated upon. Future study on the VIGS leaf tissue should be carried out to answer these interesting questions.

## 4.2 The carotenoid profiles change in response to single gene down-regulation in the petals of *E. californica* plants

The colour of *E. californica* flowers is carotenoid based and varies from white, yellow, orange to red (Wakelin et al. 2003; Barrell et al. 2010). Such variation is derived from differential accumulation of the carotenoid metabolites. White flowers of *E. californica* do not contain carotenoids in their petals or pollen (Wakelin et al. 2003). Esterification of carotenoids is common in chromoplasts (Camara et al. 1995) to enable their sequestration (Yamamizo et al. 2010), and this is also the case in the petals of *E. californica* plants used in this study. Because of this, saponification was found to be a necessary step to achieve good resolution in the HPLC analysis, which removes the attached ester groups to enable better separation of the peaks.

In this study, four major and seven minor carotenoids have been detected with HPLC in the orange flower petals of *E. californica*. They are similar to those previously reported for this flower. Maoka et al. (2000) found eschscholztxanthin and its isomers were the most abundant carotenoids found in the orange flowers, making up *ca* 35% to 40% of the total carotenoid content. Maoka et al. (2000) also identified *retro*-carotene-triol, mutatoxanthin, lutein, neoxanthin, and violaxanthin in the petal although their relative proportions were slightly different from those detected in this study. In contrast, the carotenoid profiles found in this study are different from that reported by Wakelin (Wakelin et al. 2003). The previous research found that neoxanthin and antheraxanthin were the two most abundant carotenoids. These differences highlight the large differences in carotenoid composition that can underly similar coloured petals in the natural population.

A carotenoid profile reflects the relative contributions of carotenoid biosynthesis and degradation in the cell (Zhu et al. 2010). Apart from TRV-*lcyb*, HPLC revealed distinct changes in the carotenoid profiles following single gene VIGS. qRT-PCR analysis confirmed

that the transcript abundance of the targeted genes was reduced to an extremely low level but not the other non-targeted genes in the pathway. These results demonstrate that silencing of a specific gene in the carotenoid pathways can result in blockage of the pathway in terms of metabolite accumulation. As a result, the carotenoid metabolites downstream of the blockage fail to form, but those prior to the blockage accumulate to produce a new profile. This illustrates how a reduction of a single gene transcript in the pathway leads to differential accumulation of carotenoids. This differential transcript accumulation is likely to be responsible for the wide variety of colours seen in Asiatic hybrid lily, marigold, transgenic tobacco flowers with over-expressed carotenoid genes and colour mutants of tomato fruits (Ronen et al. 1999; Moehs et al. 2001; Ravanello et al. 2003; Ji et al. 2009; Yamamizo et al. 2010).

Silencing of the early genes in carotenogenesis had a more marked effect on plant pigmentation than silencing of the later genes in the pathway. The down-regulation of *EcaPSY, EcaPDS and EcaZDS* led to white flowers. This is in agreement with the HPLC results of carotenoid profiling, which revealed that the white flowers are greatly reduced in total carotenoid content. Such reduction is consistent with accumulation of the colourless carotenoid metabolites phytofluene and the light yellow carotenoid ζ-carotene, but fewer other carotenoids. The effect on flower colour by change in expression of an early carotenogenesis gene was reported previously. In marigold petals, colour difference was shown to be due to the variation of lutein accumulation. The dark orange variety had 6-fold more lutein in their flowers than in the golden variety. The only difference was a 1.4 fold increase in transcript abundance of *PSY* in the dark orange flowers (Moehs et al. 2001). Despite this apparently small increase in transcript abundance of *PSY*, its increase may be the cause of the elevated lutein production in the dark orange marigold flowers. Increased *PSY* transcript abundance has been linked with increased total carotenoid content in juice sacs of four orange cultivars (Fanciullino et al. 2008) and in maize (Vallabhaneni & Wurtzel 2009). Over-expression of *PSY* and *ZDS* in tobacco plants leads to increased accumulation of β-carotene in their flowers (Ji et al. 2009), which is the main carotenoid metabolite that accumulates in the wild type flower (Gerjets et al. 2007). Genetically engineered *Brassica napus* with an over-expressed either bacterial *PSY* alone or in combination with *PDS* show an elevated total carotenoid content of their seeds (Ravanello et al. 2003). The lowered carotenoid content leading to reduced colour of some squash fruit varieties may in part be due to reduced transcript abundance of *PSY* (Nakkanong et al. 2012).

Flowers of TRV-*pds*-silenced plants were white, which is in agreement with the HPLC results that show the accumulation of the colourless substrate phytofluene (rather than phytoene). In a study of *Arabidopsis*, silencing of *PDS* led to the accumulation of phytoene not phytofluene (Qin et al. 2007). There are at least two possibilities as to why phytofluene accumulated in this study. First, it could be because the desaturation steps which convert phytoene to ζ-carotene *via* phytofluene are catalysed by more than one PDS and the PDS catalysing the desaturation of phytoene to phytofluene was not silenced by VIGS. Secondly, the residual PDS has a higher affinity for catalysing the first desaturation step to phytofluene than it does for converting phytofluene to ζ-carotene causing phytofluene to accumulate with the lower enzyme concentration.

Silencing of *LCYb* in *E. californica* flowers did not cause any colour change or change in the carotenoid profile of the petals. LCY activity splits the carotenoid pathway into two branches depending on what type of ring the enzyme forms at the end of the linear lycopene molecule. Mutations in *LCY* are better tolerated by plants than mutations in genes further upstream in the pathway. Differential regulation of lycopene cyclases or mutations in the genes alter colour in maize, watermelon and cara cara orange (Fanciullino et al. 2008; Harjes et al. 2008; Bang et al. 2010). A single nucleotide polymorphism (SNP) in the *LCYb* gene of watermelon reduced lycopene cyclase activity causing accumulation of lycopene which lead the flesh to be red coloured (Bang et al. 2007; Bang et al. 2010). Similarly, reduced transcript accumulation of *LCYb* increased the lycopene content of cara cara oranges (Fanciullino et al. 2008). The lack of flower colour phenotype in *E. californica* and lack of effect on the carotenoid profile as determined by HPLC may be due to the presence of multiple *LCYb*s in the flower which function redundantly, In common with what has been reported in sweet orange (Chen et al. 2010a). It may also be that the gene targeted was not *LCYb* indeed, despite its sequence similarity. This could be tested by performing colour complementation in *E.coli* as described by Shumskaya et al. (2012).

The down-regulated *CHYb E. californica* flowers had altered flower colour and significantly reduced total carotenoid content. The accumulation of β-carotene in the *CHYb*-silenced flower petal clearly demonstrated that the *CHYb* sequence cloned in this study is indeed a β-carotene hydroxylase. The fact that β-carotene was not detected in the wild type flower petals also points to the high efficiency of this enzyme. Interestingly, silencing of *CHYb* in potato tubers elevated both β-carotene content and the total carotenoid content of the tubers (Diretto et al.

2007b). This presumably is because carotenoid production in dark-held tuber chromoplasts is different to light-exposed petal chromoplasts. Other studies have also shown that changes in transcript abundance of *CHYb* leads to dramatic changes in floral pigmentation. For example, Japanese morning glory (*Ipomoea nil*) flowers are usually white, but some *Ipomoea* sp have yellow petals and this has been associated with higher *CHYb* expression in these petals (Yamamizo et al. 2010). Similarly, the total carotenoid content of interbred squash lines was positively correlated with increased *CHYb* transcript abundance (Nakkanong et al. 2012). The carotenoid content of different Asiatic hybrid lily tepals correlated most closely with *CHYb* transcript abundance when compared with any other carotenoid biosynthetic gene (Yamagishi et al. 2009). Interestingly, it was found that it didn't alter colour by just changing total carotenoid content, but rather through changing the relative proportions of individual carotenoids. For example the main carotenoid in tepals of red lily flowers is capsanthin, whereas in pink lily flowers it was a combination of violaxanthin, lutein, antheraxanthin, and b-carotene (Yamagishi et al. 2010).

ZEP silenced *E. californica* flowers also had an altered flower colour from orange to yellow and significantly reduced total carotenoid content. The colour change is a reflection of zeaxanthin accumulation, a result of not being able to be converted into the downstream carotenoid metabolites. The total carotenoid content of the TRV-*zep* flower was 20% that of the orange, indicating that the ZEP enzyme activity is important for the high carotenoid content in the petals. Very few studies in the literature have examined the effect of reduced ZEP expression. Interestingly, a developmental study of maize endosperm showed that carotenoid accumulation increased as ZEP transcript abundance decreased (Vallabhaneni & Wurtzel 2009). Therefore, more increasingly complicated regulatory elements may be involved in controlling carotenoid content of tissues.

The regulation of carotenoid biosynthesis and the resultant profile appears dependent on the relative activities of the enzymes in the pathway. Many studies, for instance, have reported the combinational effect of *CHYb* and *ZEP* in causing zeaxanthin accumulation. For example, the orange flesh of potato tubers was caused by zeaxanthin accumulation, which itself was dependent on both up-regulation of *CHYb* and down-regulation of *ZEP* (Wolters et al. 2010). By contrast, accumulation of β,β-xanthophyll in the juice sac of four orange varieties was positively correlated with increased transcript abundance of both *CHYb* and *ZEP* (Fanciullino et al. 2008). It therefore appears that the transcript abundance ratio of specific carotenoid

biosynthetic genes is important for determining the carotenoid profile and this would presumably be the case for *E. californica* flower petal colour as well.

The major end products of the carotenoid pathway in the flower petals of orange *E. californica* are two retro-carotenoids eschscholtzxanthin and *retro*-carotene-triol. Their abundance was dramatically reduced in all silenced lines except TRV-*lcyb*. Finding that their content was reduced in the TRV-*zep* plants indicated that epoxidation was required for their synthesis. This is consistent with a previous study in *Narcissus pseudonarcissus*, which showed that retro-carotenoids are produced from violaxanthin (Lutzow et al. 1996). The accumulation of *retro*-carotene-triol was reduced to a greater extent than eschscholtzxanthin in TRV-*chyb*, which is consistent with eschscholtzxanthin being produced from *retro*-carotene-triol as suggested by the work of Maoka et al. (2000). Because retro-carotenoids are red in colour (Hormaetxe et al. 2005), the orange colour of the *E. californica* flower could be due to the mixing of the yellow and red carotenoids. This would be consistent with the finding of a loss of retro-carotenoids in the HPLC profiles of the yellow flowers. Comparative analysis of the flower gene profile and colour profile of the yellow, orange and red varieties may give more insights into the particular genes that are responsible for the retro-carotenoid synthesis.

## 4.3 Carotenoid metabolites do not appear to work as feedback regulators of carotenogenesis transcription

Previous literature has suggested the carotenogenesis pathway is positively feedback regulated by accumulation of its metabolites (Corona et al. 1996; Al-Babili et al. 1999; Sun et al. 2010). In this study, each targeted carotenoid biosynthetic gene was successfully silenced by VIGS causing the substrate of the protein encoded by the targeted gene to accumulate. However, none of the accumulated substrates, except for perhaps β-carotene feedbacked to differentially alter transcript abundance of the other genes in the biosynthetic pathway. The increase in β-carotene resulting from VIGS of *EcaCHYb* gave rise to a slight fold increase in *EcaLCYb* transcript abundance. None of the other transcripts reached this 2-fold threshold. The effect of β-carotene accumulation on *EcaLCYb* transcript abundance needs to be confirmed in subsequent studies. Overall my findings are more in line with a recent study on an EMS mutant of tomato, which showed no feedback regulation of the transcript abundance

of the carotenoid biosynthetic pathway in the fruit when the *PSY1* gene was knocked out (Gady et al. 2012).

Some studies that have over-expressed certain enzymes in the carotenoid biosynthetic pathway also have provided no evidence for feedback on the transcription of the pathway. For example, over-expression of *LCYb* in tomato fruit, which converts lycopene to β-carotene and lowered the total carotenoid content also did not significantly change transcript abundance of any of the other tested endogenous biosynthetic genes (Giorio et al. 2007). Over-expression of the bacterial *PSY* and *CrtI*(*PDS*) genes in golden rice, which elevated the total carotenoid content, and resulted in increased β-carotene content, again did not alter transcript abundance of the endogenous biosynthetic genes (Schaub et al. 2005). Similarly, *CrtB(PSY)* over-expression in potato tubers enhanced carotenoid content and altered the carotenoid profile, however the abundance of the endogenous carotenoid gene transcripts remained unchanged (Ducreux et al. 2005).

In contrast to the above studies, others have suggested that transcript abundance is affected by altered metabolite content. For example potato transformed with the bacterial carotenoid genes *PSY*, *PDS* and *LCYb* substantially increased the total carotenoid content especially β-carotene and up regulated the endogenous carotenoid genes in the tubers (Diretto et al. 2007a). Feeding of green alga with excess amounts of GGPP, the precursor of carotenoid biosynthetic pathway caused an increased transcript abundance of *PDS*, *LCYb* and *CHYb* (Sun et al. 2010). The *LCYb* mutant maize kernel contained increased amounts of total carotenoids and showed an elevated transcript abundance of most of the carotenoid genes. This led these researchers to propose that a regulatory metabolite downstream of lycopene regulated carotenoid transcription (Bai et al. 2009). Similar situations were found in red-fleshed papaya, with a mutation in one of the *LCYb* genes leading to a doubling of the total carotenoid content and accumulation of lycopene (Devitt et al. 2010; Schweiggert et al. 2011).

There are a number of potential reasons for the apparent discrepancies highlighted in above studies. The effect of oxidative damage in photo-bleached photosynthetic tissues by the silencing of the early carotenoid genes may mask the actual regulation of the pathway. A functional chromoplast structure could be required for the pathway feedback to take place. It may be that the chromoplast ultrastructure more tightly regulates the pathway rather than the accumulation of metabolites. Or there may be a carotenoid accumulation threshold needed for

regulation of the pathway. Finally, the carotenoid biosynthetic pathway regulation may occur initially at the enzymatic or posttranscriptional level rather than transcriptional.

Where carotenoid content has been substantially reduced, the cells potentially would experience large amounts of photooxidative stress. This can potentially confound analysis by make it difficult to determine whether the response is due to a lack of carotenoids or due to a general stress response. Therefore, consideration needs to be taken as to whether the carotenoid feedback regulation is the primary cause of carotenoid gene expression alteration. Also, as carotenoids are synthesised and stored in chromoplasts, functional chromoplasts may be integral to the regulation of carotenoid biosynthesis. For example, the *pds3* mutant of *Arabidopsis* was found have increased phytoene accumulation and decreased carotenoid gene expression in terms of transcript abundance. This was suggested to be because of metabolic feedback regulation of the carotenoid pathway (Qin et al. 2007). However, the strong photobleaching phenotype of the *pds3* plants indicated that severe oxidative damage had occurred in the plants, which presumably means multiple pathways in the plant would have been affected and would make it difficult to conclude that direct feedback by the carotenoid pathway had occurred. In addition, the failure of normal chloroplast development would also raise questions as to whether the carotenoid feedback was detectable and taken place in plants under such a high level of stress.

Ultrastructural changes in the chromoplast which directly related to the sink size of carotenoid accumulation may be more important for controlling carotenogenesis than carotenoid metabolites. Further studies have revealed the relationship between change of the carotenoid composition and change in the chromoplast ultrastructure. Yellow daffodil flowers treated with a LCY inhibitor, CPTA [2-(4-chlorophenylthio)triethylamine hydrochloride] turned the flower colour from yellow to red. This was due to a doubling of the total carotenoid content in the red flower and increased accumulation of lycopene over the normally produced lutein. Carotenoid genes in the CPTA-treated daffodil flowers were up-regulated both at the transcript and protein level (Al-Babili et al. 1999). These researchers also recorded that the chromoplast ultrastructure changed after treatment with CPTA and accumulation of lycopene. This was also observed in canola seeds where *CrtB*(*PSY*) over-expression not only caused induction of carotenoid accumulation but also alteration in chromoplast ultrastructure (Shewmaker et al. 1999). Furthermore, over-expression of *PSY* in tomato caused alteration of the chromoplast ultrastructure and up-regulation of the carotenoid pathway both at the

transcript and enzyme activity level (Fraser et al. 2007). The tomato plants carrying a mutant *ZEP* gene had increased carotenoid content and an increased number and size of chromoplasts (Galpaz et al. 2008). Together, the evidence points to carotenoid storing capacity playing an important role in controlling carotenogenesis.

The change of chromoplast ultrastructure could be dependent on the total amount of carotenoid and accumulation of certain metabolites. The form of carotenoid deposition seems dependent on the amount and type of carotenoid present. For example, small amounts of lycopene are stored in osmiophilic globules, whereas large amounts are stored in the crystalloid form (Simpson et al. 1974). The accumulation of a different carotenoid may also modify the chromoplast ultrastructure. The yellow–flesh papaya contains tubular chromoplasts, whereas the red-fleshed papaya which is enriched in lycopene has more tomato-like crystalloid chromoplasts (Schweiggert et al. 2011). The roots of *Arabidopsis* plants over-expressing *PSY* had increased β-carotene content in crystals, which resembled crystalloid chromoplasts (Maass et al. 2009).

The regulation of carotenogenesis may occur at multiple levels, i.e, at the enzyme activity, protein accumulation and gene transcript level. The majority of research on carotenoid feedback regulation has focused on the transcriptional control. However, some studies have shown that carotenoid gene expression and protein abundance does not accurately represent the enzymatic activity of the pathway. For example, tomato plants transformed with *CrtB* showed changes in carotenoid composition including increased β-carotene content, but a reduced (50%) total carotenoid content. The transcripts of endogenous genes showed less than 2-fold changes as judged by qRT-PCR but surprisingly, PDS enzyme activity increased 8-fold (Roemer et al. 2000). Similarly, when the enzymatic activities were present along with transcript abundance for PSY over-expression tomato, the change at the enzyme activity level did not all agree with the change of the transcripts abundance (Fraser et al. 2007). Both active forms and inactive forms of PSY protein were discovered that are membrane-bound and non-bound respectively (Welsch et al. 2000). The active and inactive forms of other carotenoid enzymes were also found such as PDS, CRTISO (Lopez et al. 2008b; Yu et al. 2011). Therefore the regulation of the carotenoid biosynthetic pathway occurs at multiple levels, which includes both altering transcript abundance and enzyme activity.

## 4.4 Summary

This study has provided little evidence to support the hypothesis that transcriptional control of carotenogenesis is under feedback control by metabolites in the pathway. No dramatic change in any carotenogensis transcript was observed when the carotenoid metabolite profile was substantially changed in numerous ways. These changes included significantly reducing end products (e.g., retro-carotenoids), and the preferential elevation of individual metabolite content over that seen in wild type (e.g. zeaxanthin). It should be noted however, that in all cases overall total carotenoid content was lower in the silenced lines so we cannot rule out whether elevated total carotenoid content might affect transcriptional regulation.

This study has also highlighted for the first time that phytofluene rather that phytoene accumulates in the TRV-*pds* flowers when there is residual PDS activity. Also of note was the finding that in *ZEP*-silenced petals, zeaxanthin was the only carotenoid that was of significant abundance. If this is representative of what is happening in leaves then this would suggest that even a single carotenoid structure present in the leaves is enough to prevent photobleaching. This is worthy of further study. In addition, this study suggests that if carotenogenesis is not regulated through metabolites at the transcriptional level then so it may be through post-transcriptional means.

Thus a new regulatory model for the carotenoid biosynthetic pathway is proposed based on the findings in the thesis and the literature. The biosynthetic pathway may be feedback regulated by a change in chromoplast ultrastructure, which occur first at the post-transcriptional and then at the transcriptional level. The modification of chromoplast structure could be caused by changes in the carotenoid composition and total amount, and changes of the carotenoid profile may initially respond to alteration of the carotenoid biosynthetic enzyme activities. The further increased accumulation in carotenoid content and increased enzyme activities may eventually alter the chromoplast ultrastructure. The more powerful regulation may be activated by feedback control of the expression of carotenoid biosynthetic genes by ultrastructural changes to accommodate the dramatic change in carotenoid composition.

To answer the remaining questions and test the new model, future research will need to be undertaken to assess the change of carotenoid biosynthetic enzyme activity and examine chromoplast ultrastructure in TRV-infected flowers. Both carotenogenesis enzyme inhibitor-treated plants and gene over-expression plants should also be studied for their carotenoid biosynthetic enzyme activities and gene expression profile along with chromoplast ultrastructure and carotenoid profile. The ultrastructure change could be resolved by microscopy and qRT-PCR testing of genes encoding chromoplast structural proteins such as fibrillin.

# Appendix 1

# Recipe for buffers, media and solutions

## 1.1 Bacterial media

### Luria-Bertani (LB) Broth and media

(Sambrook and Russell, 2001 Gerhardt, *et al.* 1994).

| | |
|---|---|
| Tryptone | 10 g |
| Yeast Extract | 5 g |
| NaCl | 10 g |
| Water | 1 L (final volume) |

For LB agar, add agar to a final concentration of 1.5%.(w/v)

Heat the mixture to boiling to dissolve agar and sterilize by autoclaving at 15 psi

## 1.2 Plant growth media

### Daltons - Crop & Food pH mix ingredients for 1 $m^3$

| | |
|---|---|
| C.A.N Fines A Grade | 50% |
| Fibre | 30% |
| Pacific Pumice | 20% |
| Dolomite | 2.00 $Kg/m^3$ |
| Gypsum coarse | 1.00 $Kg/m^3$ |
| Hydroflo II G Wetting Agent | 1.00 $Kg/m^3$ |
| Lime | 2.00 $Kg/m^3$ |
| Serpentine Super | 0.50 $Kg/m^3$ |

Added Fertilisers

| | |
|---|---|
| Osmocote plus 8-9mth | 2.7 Kg |
| Dolomite | 1.0 Kg |
| Superphosphate | 300 g |
| Potassium Sulphate | 300 g |
| Calcium ammonium nitrate | 120 g |
| Iron | 180 g |
| Micromax | 60 g |
| Gypsum | 200 g |
| Terrazole | 60 g |

### 1.3 Electrophoresis reagents

**10 x TBE buffer**

| | |
|---|---|
| Tris-base | 0.89 M |
| Boric acid | 0.89 M |
| EDTA | 0.3 M |

**10 x MOPS Buffer**

| | |
|---|---|
| MOPS | 0.2 M |
| Sodium acetate | 50 mM |
| EDTA | 10 mM |

Make up with water to 1 L and adjust to pH 7.0 with NaOH, Autoclave

**1% (w/v) agarose gel**

| | |
|---|---|
| Agarose | 2.5 g |
| 1xTBE buffer | 250 mL |
| Ethidium bromide (10 mg/mL) | 5 μL |

Set at room temperature for 15 min

**10 x loading dye**

| | |
|---|---|
| Glycerol | 30% (v/v) |
| EDTA | 0.1 M |
| SDS | 1% (w/v) |
| Bromophenol blue | 0.25% (w/v) |
| $H_2O$ | up to 10 ml |

**1.2% (w/v) MOPS agarose denaturing gel**

| | |
|---|---|
| Agarose | 1.95 g |
| 10 x mops buffer | 15 mL |
| Water | 150 mL (final volume) |

Melted in microwave and allowed to cool to 50 ˚C

| | |
|---|---|
| Formaldehyde | 7.65 mL |

Set in fume hood at room temperature for 15 min, run in fume hood

**2 x RNA denaturing solution**

| | |
|---|---|
| 10 x MOPS | 500 µL |
| Formamide | 500 µL |
| Formaldehyde | 150 µL |
| Ethidium bromide (10mg/ml) | 5 µL |

Stored in foil-cover tubes at -4 ˚C

**1.4 Alkaline lysis miniprep solutions**

**Solution I- re-suspension buffer**

| | |
|---|---|
| Glucose | 50 mM |
| EDTA | 10 mM |
| Tris pH8 | 25mM |

**Solution II- lysis solution**

| | |
|---|---|
| SDS | 1% (w/v) |
| NaOH | 0.2 M |

**Solution III- neutralisation solution**

| | |
|---|---|
| Potassium acetate | 3 M |
| Glacial Acetic Acid | 8.7% (v/v) |

**1.5 Plant infiltration**

**Infiltration buffer**

| | |
|---|---|
| MES | 10 µM, |
| $MgCl_2$ | 10 mM |
| Acetosyringone | 150 µM |

**1.6 RNA extraction**

**Hot Borate total RNA extraction buffer**

| | |
|---|---|
| Sodium borate decahydrate | 0.2 M, |
| EDTA | 30 mM |

| | |
|---|---|
| SDS | 1% (w/v) |
| Deoxycholate, sodium salt | 1% (w/v) |
| DTT | 10 mM |
| Nonidet P-40 | 1% (v/v) |
| soluble PVP | 2% (w/v) |

### 1.7 Carotenoid extraction

**Carotenoid extraction buffer**

| | |
|---|---|
| Acetone | 140 mL |
| Methanol | 60 mL |
| $CaCO_3$ (when added) | 40 g |

### 1.8 Antibiotics

**Ampicillin (100 mg/mL)**

| | |
|---|---|
| Sodium salt of ampicillin | 2 g |
| milliQ water | 20 mL |
| store at -20 ˚C | |

**Kanamycin (100 mg/mL)**

| | |
|---|---|
| Kanamycin sulfate | 2 g |
| milliQ water | 20 mL |
| store at -20 ˚C | |

**Gentamicin (60 mg/mL)**

| | |
|---|---|
| Gentamicin sulfate | 1.2 g |
| milliQ water | 20 mL |
| store at -20 ˚C | |

**Rifampicin (100 mg/mL)**

| | |
|---|---|
| Rifampicin | 2 g |
| milliQ water | 20 mL |
| store at -20 ˚C | |

# Appendix 2

# Carotenoid biosynthetic gene sequences and primer locations

The carotenoid biosynthetic genes sequence fragments obtained from search an *E. californica* petal 454 sequence data by Dr Mark Fiers (Plant & Food Research). The primers for amplification of sequence for VIGS was coloured in orange, the qRT-PCR primers for determining native gene expression levels are coloured in green.

*EcaPSY*

GAATTCGATTGAAGGAATGCGAATGGACTTGAGGAAATCAAGATACAAGAACTTTGATGAAC
TCTATCTGTACTGTTGTTATGTAGCAGGAACTGTTGGATTAATGAGTGTCCCTGTAATGGGA
ATTGAACCAGAATCTCAAGCAACGACGGAGAGTATCTATAGTGCTGCTTTGGCGTTAGGAAT
TGCAAATCAACTCACAAATATTCTTAGAGATGTCGGAGAAGATGCAAGAAGAGGAAGAGTTT
ATCTACCACAGGATGAGCTAGCACAGGCAGGGCTTTCCGACGAAGACATATTCGCAGGCAAG
GTAACTGATAAGTGGAGGAGTTTCATGAAAAAACAGATACAAAGGGCACGGATGTTCTTCGA
CGAAGCAGAGAAAGGAGTAACACAACTCAGCTCAGCGAGTAGATGGCCGGTGTGGGCATCTT
TGTTACTGTATCGCCAAATACTAGACGAGATCGAAGCAATCACTAGTG

*EcaPDS*

TCGTAATACTCCTTACATGTTACAGACATGTCAGCATATACACTCAAGAGGGGACTTCTGCT
GAAGAGTAGATGATCATACGTGTTTTTCAGTTTTCTGTCAAACCATATATGAACATTAATCA
CAGGAACTCCAACTAATTTGTCCAATCTCTTGAAGTACGGAACTTCTTTCCATTCTTCAGGT
AAAAGAAGCTTCAGGATGTCAACTGGTGCTGCGATTACATAGGCATCTCCTTCTATTGCATC
ACCATTAGTAAGTATAAGACGCTTCACACTTCCATCACTTTTCAGATCAATCTTCTGAATCC
GTGAATTTAGATGGACTTTGCCACCAAGTGATTGGATGTGATTAACTATCGGCATGCAGAGT
CTCTCAGGAGGATTACCATCTAAAAAGGCCATTTTGGAACCATGCTTTTCCTGAAGAAATCG
GTTCAAAGCTATCAAAATGCACTGCATTGAAAGCTCGTCTGGGTTTATGAAGTTTAGTGCCT
TCGACATCGCGATAAACACCTCATCAGTTACTCGATCAGGTATACCCTGCTTTCTCATCCAA
TCTTTCACAGTTAAGCCATCTTGAGCCTCCACGTAAGGCTGTCCTCCAAGCATTGCTGGCAA

GAGTCCGATGGCAAACCGTACTTTCTCTGGCCACGTAAGCATTTCATTGTTTCTTAAGATGG
CCCAGATTCCGTTTAAGGGTGCTGGAAGAACGTCAGGGAAATCAAACCGGCTGAATTCTCCT
GGCTTGCTTGGCATGGCAAAAATCATGGAGTGTTCCTTCCACTGCAGCCTGTCATTTATACC
AAGTTCTCCAAATAAATTCTGCACGTTCGGGTATGCTCCGAAGAATATGTGTAAGCCGGTTT
CATACCAGTCCCCATCGTCATCCTTCCATGCAGCTATCTTTCCACCCAAAACATCTCTTGCT
TCTAGCAATATGGGCTTGTGACCTGCATCTGCCAGATACTTTGCTGTGGATAGACCAGCCAA
GCCTGCACCAGCAATTATAACTTGTAATGGTTTTTTGGGGCGGACGGAAGTACTGAAGGACG
ACGAAAAATACGCTGCCTCCAAGAAATTAACAGTATTATCAATTTCAGGCCTCGGATAGTCC
ATGCAAACAACCTGCAAAGGGCCTATCTTCCTCCTTGCCCGACTTCCGACAGAATGTGCCTT
TGGAGATTCTAGGGACTGACCCATAGAATGACTCCCTCCAAATGCTAATAGACTAGTATTAT
CTCTATATTTTGTAGAAAAACACCATCTTCTAGTGGTATTTGAGCTAGAAATATTCATCATA
TTACTTAACCCACTCAAATTTACAACAGATACAGTACCAGCTACAGTCATCTCCGCCAATTT
CAATCACCAAATTAGTTATACAACAAGAATTACCAAAACCCACTCTGATGAGCCTGCATATG
TCCACAATTCAGAGCATATACGCCCCAGTGGCTCAGCAGAGCTTGAAGGAGTGAAATCTTTA
ATCTGG

*EcaZDS*

TACGCTCTGGGTTTTATTGACTGTGACAATATGAGTGCCCGTTGCATGCTAACTATATTTGC
ACTATTTGCCACTAAGACAGAGGCTTCGTTACTCCGCATGCTTAAGGGATCTCCTGATGTTT
ATTTGAGTGGCCCTATCCGAAAGTATATAATAGACAAAGGTGGCAGGTTTCATCTCAGGTGG
GGTTGCAGAGAGGTGCTTTATGATAAATCTGTCAATGGAGACACTTTTGTTTCAGGACTTGC
CATGTCTAAGGCTACAGACAAGAAAATAGTAAAAGCTGATGTCTATGTTGCAGCGTGTGATG
TTCCGGGAATCAAAAGGCTAATTCCATCAGAGTGGAGAGAGTGGGAGTTGTTTGACAACATT
TACAAACTAGTAGGAGTTCCTGTTGTCACTGTTCAGCTAAGATACGATGGCTGGGTCACAGA
ATTACAAGACCTCGGAAAGTCAAGGCAGTCGAAGAAAGCTGTAGGGTTGGACAATCTGCTTT
ACAGTCCAGATGCAGATTTTTCCTGTTTCGCTGATCTCGCACTCACGTCTCCGGAAGATTAT
TATATCGAGGGTCAAGGTTCATTACTCCAATGTGTGCTTACACCTGGGGATCCCTATATGCC
ATTAACGAACGATAAGATCATTGAGCGAGTGGCGAAGCAGGTTTtGACTTTATTCCCATCTT
CTCAAGGCTTGGAACTTATTTGGTCTTCTGTTGTTAAAATCGGGCAATTCCTTTACCGGGAA
GGACCTGGAAAAGATCCTTTCAGACCTGATCAAAGGACACCCGTGAAAAATTTCTTCCTCGC
TGGGTCTTACACAAAACAGGATTACATCGACAGTATGGAAGGAGCA

*EcaLCYb*

GAATTCGATTTAGAAAGCCGTGCCAATAATAAGGATCTAGACTAAAGAAAGCATCAAAAAAC
CTTCTTGTTCCCTTCAAATCAAGCTTCAAAAGAGTCTCCATTCCAAAACAATAAATATCTCT
ACTACATCTCTTATCCAATGGCCACAATCTATCCCAAACTCTTTGATGTAATTGAGACCCTC
TAATCATCCTAGTGGACCCTAAACACTCGTAGATCACGTCCGCGAGTACTGTCGATCCGGCC
AAAGCTTGAGCTACCATGTATCCAGTTGAAGGGTGAACCAATCCTGCACTTGCACCATAACA
CATTAGACTCTGTGGTATAGTAGGAAGAGGACCTCCCATTTGAATTTGACATCTTTCATCTT
CTATGATTCTCTTCACTTTGATACCCAAATGTCTTAACCTTGCTACCATCCTATTCTTCACT
TCATTATGTGRTAAAGCTGGTCTACTCACTAATGATGTCTCTTCGAGAAAAATCAAATTCCG
ATCGATTGGCATTGCGTATAAGAATGTAGGGAACTTGTTGTTATTAGCACGTAAAATTGGTT
CATTACCCATATGGGTATCTCTCCAATCCATTAAAACCATTTTATCCAAATCAAATRGGTGT
TCATTTACCTCTGCTAATATACCATGAGCAATTTGATATCCATGGTTTCTTGGTCTATTGTA
CTTCACAAAAGAACTTTGAAACCCACTTGCATCAACAATCAAACTTCCTTTTAATTCGATTC
CATCTTGACATACAACAATAGATTCAAATTCTTGGTTCAAAATCTCACATACTTTCGCTTTG
AAAAACTTCACTCCATTTGAAACACAACTTTCTACCAACTTTGTTTTCAATAGATTCCTACT
AACTCGCCCGTAAGGACGGTCGAGATACTTAGCTTTTCGATCATCAATATAGATTTTAGCCA
TTGCCCATTTCTTGTCTAAACAATCTTCAAGGCCTAGACTTTGAAACTCATCCTCCAAACAC
CATAATTGTTAATCACTAGTG

*EcaCHYb*

AAATTGATTGAAACCTTCCATTACAGATTTCCATGACTATCTTAAATCCACATTTTGCATTC
AATTCATCCCTTAGATTCCCCAGGTTCTGTTTCTCACTTTCTCTCTCTAACTTAATTCTTAT
TTCTTTCTCTCTCTAGGACCTTTTTCTTCTTCTTATAAAGTCTCTTCTCCCTCTGTTTACAA
AAAATTGAATCTTCAAAACAGAGAGTTTCCATGGCGGCTGGGTTCTCTGTCGCTTCAACCTC
AAGAACTTTCAGTTTTGGACAAAATAAGCTTTTTTGTTCAAAACCCAATTCTTTAATTACTT
CTTCTTCCTTTCTATCTCTACCAATTCGTAGACCCAATCAAAATTTATCTACTACTAATAAG
AGAAAGAATTGTTTTACTGTTTATTTAGTATTGGGGGAGAGAAAACAGGGGATTAGTAATGA
AAATAAAGAAGATAAAGTTGGAGAAATTTCAACAGAATTGAATCAGATTTCAACTAGAATTT
CTGAGAAATTAGCTAGGAAGAAATCAGAAAGAACTACTTATCTTGTTGCTGCTATTATGTCT
ACTGTTGGAATTACTTCTATGGCTATTGCTGCTGTTTATTACAGATTTTCATGGCAAATGGA
GGGTGGAGAGATTTCTCTTTCCGAAATGTTTGGTACATTTGCTTTATCAGTAGGAGCTGCTG
TTGGGATGGAATTTTGGGCAAGATGGGCTCATAGAGCTCTTTGGCATGCTTCTTTATGGCAT
ATGCATGAGTCTCATCATCAACCAAGAGAAGGTCCATTTGAATTGAATGATGTTTTCGCCAT
TATAAATGCTGTTCCTGCTATTGGTCTTCTCTCTTATGGGTTCTTTCATAAAGGCCTCTTTC

CTGGTCTCTGTTTTGGTGCTGGACTTGGAATTACATGTTTTGGAGTAGCATATATGTTTGTT
CATGATGGTCTTGTTCACCGTAGATTTCCAGTCGGACCCATTGCTAATGTTCCTTACTTCCG
AAAAGTTGCCGCAGCACACAAAATTCATCACATGGACAAATTTAATGGAGTACCTTATGGGT
TGTTTCTAGGACCAAAGGAATTGGAAGAGGTGGGAGGTTTAGAAGAATTAGATAAAGAAATC
AATCGTAGAATCAAACAATTCAGTGGTCCAAGATGAAATTGTTGAGGTGGGGTTAACCCTTA
ATGTTACATTATGGTGTGATTAAAGATTTTGGTTGTATGTAGATCAAGCAAAAAAGTATAAA
AATTAGAAAAATATTAAGTGATAAGTCCATAAAGATGGATTAT

*EcaZEP*

AAGACTACTATGAAGCAATTTTACCTTCTCTTCATTACAATAGTTCATTTAGAACCAAACCC
TTTGAGCATAGAAAGAGAAAACAAGTTAAAATTCTGCAACAACATCAATACCAACTACTCAA
GAAACTGATTCTTCCCCCAAAAGTGGAAGTGGGGATCCAAAGAAACTTAGGGTTTTGATTGC
TGGTGGTGGAATTGGTGGGTTAGTTTTTGCTTTGGCTGCTAAGAGAAAAGGGTTTGATGTTT
TGGTATTTGAGAGGGATATTAGTGCTATAAGAGGTGAGGGTCAATACAGAGGACCAATTCAG
ATACAGAGTAATKCKWTWGCTGCWTTRGAAGCTATTGATTTGGAGGTAGCTGAAGAAATTCT
CAAAGCTGGTTGTATTACTGGTGATAGGATTAATGGATTAGTTGATGGCATCTCTGGTGCTT
GGTACATCAAGTTCGATACATTCACACCTGCAGTCGAACGGGGGCTTCCTGTCACAAGGGTT
ATTAGCCGAATGACCCCTCAAGAAATTTTGGCTAAGGCAGTTGGGGAAGATGTTATTAGTAA
TGATAGTAATGTTGTTGATTTTGAAGATGATGGAAATAAGGTTACTGTAATACTTGAGAATG
GAAAGCGGTTTGAAGGTGACCTTCTAGTTGGAGCTGATGGAATATGGTCTAAGGTCAGGAAG
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TTGTTTCTTCGGATGTGGGTGGTGGAAAGATGCAGTGGTTTGGATTTCATAAAGAAGAACCT
GGTGGTGTTGATGCTCCAAATGKCAAAARGGAAAGATTGTTTCAAATATTCGGGAGTTGGTG
TGATAATGTGGTTGACTTGTTAAATGCCACGGAGGAGAGTTCCATTTCTTCGACGTGACATA
TACGATAGAATACCTATCTTTTCGTGGGGAAAGGGTCGTGTAACCTTGCTTGGAGATTCCGT
TCATGCTATGCAGCCAAATTTGGGCCAAGGTGGTTGCATGGCTATTGAGAATTTTCTTGAGG
ATAGTTATCAACTCGCTYTGGAGCTCGAGAAAGCTTGGAGTGGAAGTGTAAAATCTGGAGCT
TCGGTTGATGTCRCATCTGCTTTAAAACGTTAYGAAAAAGAGAGAAGATTGCGTGTTGCCAT
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GGGAGATTTTTCATTGATCTCGGAATGCCTCAAATGCTAAGTTGGGTCCTAGGAGGTAACAG
TGGAAAACTTGAAGGAAGGTCATTAAGCTGCCGACTCTCAGATAAAGCGAATGATCAACTAA
GGAAGTGGTTTGAAGATGATGATGCATTAGAGCGCGCCATTAATGGAGAGTGGTTTCTTTTT

CCTTCGGGAAATCAAAATAACGATTCCTTTCAACCAATTCGTTTGAGTGTAGATGAAAATAA
GCCCATCGTCATTGGAAGCTCTTCACAAGCCAACCCTTCAGGATCGTCAATCGTCATACCTT
CACCTCAGATATCAGAAACACATGCGCGTATTAGTTGCAAAGATGGATTTTTTTATGTCACC
GATATGAGTAGTGAACATGGCACATGGATCACCGATATTGAGGGAAGACGCTATCGAGTCCC
TCCAAACTTTCCCGCTCGTTTTCGAATCACTAGTG

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Name of Candidate: Jun Zhou ID Number: 01259431

Degree: MSc Dept/Institute/School: IMBS

Thesis Title: Regulation of the carotenoid biosynthetic pathway in California Poppy (*Eschscholzia California*) petals

Name of Chief Supervisor: Professor Michael McManus Telephone Extn: 2577

As author of the above named thesis, I request that my thesis be embargoed from public access

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