Chapter 3

Expression of Carotenogenic Genes
SUMMARY

To study the biosynthesis of carotenoids, the pathway was first regulated by elicitation in two different coriander cultivars - GS4 Multicut and Mahak, the first having the ability to produce highest content of carotenoids and the second with low level of carotenoids, particularly with low β-carotene content. Foliar-application of elicitors, methyl jasmonate (MeJa) and salicylic acid (SA), differentially elicited total carotenoids, β-carotene, lutein, chlorophylls, total phenolics and chlorogenic acid in coriander. Carotenoids and total phenolics increased 6.8 and 3 folds respectively when treated with MeJa (10 µmol/L), whereas SA (500 µmol/L) showed 5.4 and 3.5 folds of respective compounds. These treatments also enhanced levels of β-carotene, lutein, chlorophylls and chlorogenic acid, as observed by HPLC/MS analyses. Carotenoid and phenolic extracts of the best treatments showed significant increase in hydroxyl and superoxide radical scavenging antioxidant activities compared with controls. The observations made here indicate that precise elicitation is a novel natural method for significant enhancement of important compounds in coriander. To elucidate the molecular mechanism of carotenoids accumulation, transcript expression profile of ten carotenogenic genes under the influence of MeJa was determined. In coriander, a significant difference in the degree of expression of genes after treatment is correlated with carotenoids content at different times. Application of MeJa (10 µmol/L) increased the expression level of CsPDS, CsZDS, CsCHXE and CsLCYE. However, a higher concentration (500 µmol/L) suppressed the expression of CsCHXE and CsCCD1 after 3rd day with no expression on 12th day. To elucidate these events at molecular level, regulation of carotenoids pathway genes were studied by both elicitation and by treatment with inhibitor – norflurazone, which specifically inhibits phytoene desaturase. While most of the carotenoids were enhanced after elicitation, both β-carotene and lutein were suppressed by norflurazone. In the latter, along with CsPDS other genes CsLCYE and CsZDS were also down regulated. To demonstrate the regulatory genes in the carotenogenic pathway, plants were treated with three specific inhibitors such as fosmidomycin, norflurazone and amitrol which targeted initial steps in the MEP pathway, phytoene desaturase and lycopene cyclases respectively. This study showed that, phytoene desaturase and ζ-carotene desaturase are the highly and most differentially expressed genes and their transcript levels were strongly associated with carotenoid content in coriander foliage.

Publication
3.1. Introduction

Carotenoids are lipid soluble pigments with high structural diversity and perform diverse biological functions in all photosynthetic organisms (DellaPenna 1999). Carotenoids act as light absorbers in photosynthetic membranes and offer protection against damages caused by photo oxidation (Bartley and Scolnik 1995). Carotenoid pigments of flowers attract pollinators and those of fruits attract seed dispersal agents (Howitt and Pogson 2006; Tohge et al. 2014). Carotenoids provide substrates for the synthesis of abscisic acid - the phytohormone that chiefly regulate stress in plants (Du et al. 2013; Neuman et al. 2014). Carotenoids from plant origin such as α-carotene and β-carotene have specific importance in human diet, since they serve as precursors of vitamin A, and this vitamin is important in preventing blindness, xerophthalmia, and premature death (Rahal et al. 2014; Pons et al. 2014). In addition, there is an inverse relationship with dietary intake of other carotenoids and the risk of various types of cancer (Finley 2005), cardiovascular disease, cataracts, photosensitivity diseases (Granado et al. 2003; Rahal et al. 2014), skin cancer and immune deficiency disorders (Fiedor and Burda 2014; Hughes 1999). The health benefits of carotenoids are also related to their antioxidant activity and involvement in removing reactive oxygen species that cause oxidative damage to cells. Consumer awareness is increasing about the health benefits offered by carotenoids from natural products. According to Global Industry Analysts (GIA), world market for carotenoids is projected to reach US$1.2 billion by 2018 (http://www.strategyr.com/pressMCP-1700.asp). GIA also noted that natural carotenoids were expected to find increased acceptance among consumers, where the market dominance for β-carotenes was attributed to the increased awareness about its health benefits and growing demand as a natural colorant in bakery and dairy products.

The nutritional richness of green leafy vegetables (GLVs) and other advantages such as their low cost and availability throughout the year have been described in previous chapters. Therefore, the consumption of GLVs is encouraged to alleviate health problems such as vitamin A deficiency, age-related macular degeneration, neural tube defect in infants and cataract – which are prevalent in developing countries, including India. In GLVs, it is possible to further enhance the biologically active
carotenoids such as β-carotene (a pro-vitamin A carotenoid) and lutein (an important pigment that slows down macular degeneration and cataract) as well as other pigments such as chlorophylls (known for strong anti-cancer and anti-oxidant effects) (De Vogel et al. 2005) and anti-oxidative phenolics which are known to impart health benefits. There are several types of compounds that elicit pigment biosynthesis in photosynthetic foliages, and some such compounds are also synthesized by the plant itself to regulate its own metabolism. One such group of compounds is the endogenously produced jasmonates in plants. Jasmonates are known to be actively involved in maintaining *in planta* redox homeostasis, keeping cells in an active physiological status that render protection against both biotic and abiotic stresses. In addition, the exogenously applied MeJa (elicitor) was found to enhance expressions of genes that encode stress related proteins, as well as those involved in the synthesis of secondary metabolites (Cheong and Choi 2003; Rohwer and Erwin 2008). Several studies have demonstrated that the effects of different elicitors on secondary metabolites vary in each plant and different organs of the same plant (Wang et al. 2008; Kuzel et al. 2009; Pérez-Balibrea et al. 2011; Cao et al. 2012). Therefore, the elicitor application on any organ of a plant species needs experimental validation for its practical feasibility in enhancing a particular secondary metabolite. Elicitors - MeJa (Kim et al. 2007) and SA are classified by US Food and Drug Administration as Generally Recognized As Safe (GRAS) substances, indicating their feasibility for application for the enhancement of nutritional compounds in fresh plant food commodities (Poulev et al. 2003).

Through previous chapters, one can understand the importance of coriander foliage, which is vastly used both as a vegetable and a spice, owing to its appealing flavor, medicinal properties and its richness in micronutrients such as vitamin C (160 mg/100 g FW), vitamin A (β-carotene 12 mg/100 g FW) and Vitamin B₂ (60 mg/100 g) (Diederichsen 1996). Coriander foliage is also endowed with a number of health-promoting functionalities such as antioxidant (Wangensteen et al. 2004) and antiplatelet activities (Suneetha and Krishnakantha 2005). As explained in earlier chapters, the present study has provided information about the profile of different carotenoids in foliage of commercially important coriander cultivars and that microwave-processing conditions are useful for reducing the loss of important pigments during drying as
explained in previous chapters, there is only one study of elicitation in coriander that report on the differential elicitation of folates using MeJa, SA, abscisic acid, kinetin and benzyl amino purine (Puthusseri et al. 2012; Puthusseri et al. 2013). However, till date no research data are available on the enhancement of carotenoids and other health promoting secondary metabolites such as phenolics in coriander foliage. Therefore, this chapter focuses on elucidating the pre-harvest application of different levels of MeJa and SA, for enhancing the concentration of total carotenoids, β-carotene and lutein. Simultaneous enhancement of other important anti-oxidants such as total phenolics, particularly the chlorogenic acid (the predominant phenolic compound in coriander foliage) was also tracked. Further, their bio-efficacies were screened by analyzing the antioxidant activities of extracts of phenolic compounds and carotenoids after elicitor treatment by in vitro assays, by measuring superoxide scavenging, hydroxyl radical scavenging and inhibition of DNA damage. In coriander foliage, except for petroselinic acid biosynthesis, so far no molecular information is available regarding the genes that involved in the biosynthesis of other health promoting nutrients such as carotenoids.

Carotenoids biosynthetic pathway, which occurs in plastid, has been widely studied in many plants and most of the genes that are involved in carotenogenesis have been identified and characterized in certain higher plants (Cunningham 2002). In the first steps of the carotenoid biosynthetic pathway, phytolene synthase (PSY) catalyses the condensation of two geranyl geranyl diphosphates (GGDP) to produce a C40 phytoene. Then, two desaturases, phytolene desaturase (PDS) and ζ-carotene desaturase (ZDS), add four double bonds to form lycopene (Figure 3.1). Subsequently, the pathway divides into two branches. In the ionone forming branch, a ε- and a β-ring are added to lycopene to form α-carotene, which is further used to synthesize lutein. In the other branch, two β-rings are added to lycopene to produce β-carotene, which is utilized for the synthesis if zeaxanthin, antheraxanthin, and violaxanthin. The genes encoding for almost all of the enzymes in the carotenoid biosynthetic pathway have been cloned and characterized from various plants (Kato et al. 2004; Kita et al. 2007). In particular, PSY and PDS have important roles in controlling the flux in the carotenoid biosynthetic pathway. For example, during tomato fruit maturation, increased expression of PSY and
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*PDS* is correlated with accumulation of lycopene (Cunningham 2002) and total carotenoid content.
Figure 3.1. Carotenoid biosynthetic pathway in higher plants. *IPI*, isopentenyl pyrophosphate isomerase; *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *ZDS*, ζ-carotene desaturase; *CRTISO*, carotenoid isomerase; *LCYB*, Lycopene β-cyclase; *LCYE*, Lycopene ε-cyclase; *CHXB*, β-ring carotene hydroxylase; *CHXE*, ε-ring carotene hydroxylase.
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The expression levels of carotenoid pathway genes and the amount of carotenoids in each plant species vary during developmental processes as well as in response to biotic and abiotic stresses (Alquezar et al. 2008). This characteristic feature points towards the presence of a highly coordinated regulatory mechanism for driving the carotenoid biosynthetic pathway and their accumulation. As stated above, certain hormones in plants, in particular methyl jasmonate (MeJa) and salicylic acid (SA) play crucial roles in orchestrating stress responses in plants. Therefore, the first set of experiments were on screening the efficacies of different elicitors (MeJa and SA) at different concentrations and the second part of the study was focused on the application of the efficient elicitor – the MeJa for elucidating the significance of each regulatory step of the carotenoid biosynthetic pathway. Using MeJA as an elicitor, the differential expression of carotenogenic genes mainly CsPDS, CsZDS, CsCCD and CsCHXE were followed, while also correlating the transcript levels of ten genes and the concomitant accumulation of major carotenoids as well as chlorophylls, since the latter are also regulated through the same pathway.

3.2. Materials and methods

Based on our earlier study (Divya et al. 2012), the highest and the least carotenoids-yielding varieties of coriander, cv. GS4 Multicut and cv. Mahak respectively, were selected. Certified seeds of these two coriander cultivars (cv.) GS4 Multicut and Mahak were sown in plastic pots (10 × 20 cm) using a mixture of soil containing equal proportions of manure, red soil and sand. Plants were raised in the greenhouse available at Central food technological research institute, under a set of conditions described earlier (Divya et al. 2012). The average light intensities were 2000 lux at 9 am, 2550 lux at 11 am, 2650 lux at 2 pm and 350 lux at 5 pm, and the atmospheric temperature ranged from 18 to 30 °C. All experiments were done keeping six sample-replicates in each and the experiment was repeated thrice.

3.2.1. Elicitor treatment on foliage

For elicitor treatment, 35 days old plants were chosen, since the rapid growth and highest biosynthesis of pigments occurred during this period. Selection of elicitors and
their concentrations were made by conducting a pilot screening experiment. Since MeJa is known to vaporize quickly, foliar applications were done in separate chambers. Each elicitor (MeJa and SA) was dissolved in distilled water containing 0.2% ethanol (v/v) to obtain four different concentrations (10 \( \mu \)mol/L, 100 \( \mu \)mol/L, 500 \( \mu \)mol/L, 1000 \( \mu \)mol/L) and applied as a single foliar spray using a hand atomizer. Control plants were sprayed with water containing 0.2% ethanol only. The plants were covered with polythene bags immediately and left for two hours, after which they were uncovered and maintained in greenhouse conditions (~16 h photoperiod) with good ventilation. A known quantity of foliage was collected after 1\(^{st}\), 2\(^{nd}\), 3\(^{rd}\), 7\(^{th}\) and 12\(^{th}\) day of elicitor treatment, as well as from control plants.

3.2.2. Extraction and estimation of pigments

Extraction of carotenoids from coriander foliage and HPLC analysis were done according to Rodriguez-Amaya and Kimura (2004) using 1 g of sample, as previously described (see Chapter 2). Identification of chlorophyll a and b were done by comparing the spectra of respective peaks with literature data, and they were quantified by spectrophotometric method.

3.2.2.1. Estimation of carotenoids by spectrophotometric method

Total carotenoid content in the extract was estimated spectrophotometrically according to Rodriguez-Amaya and Kimura (2004) which has been widely used including coriander (Guerra 2005), upon improvements made over earlier methods (Britton 1995; Rodriguez-Amaya 1999).

\[
\frac{A \times \text{volume (mL)} \times 10^4}{A_{1\text{cm}}^{1\%} \times \text{sample weight (g)}}
\]

Where A = absorbance (which was maximum at 450nm); volume = total volume of the extract; \( A_{1\text{cm}}^{1\%} \) = absorption coefficient of \( \beta \)-carotene in PE (2592). Here we used \( \beta \)-carotene for comparison, which is a common practice to measure such pigments, similar to the use of cyanidin-3-glucoside for the estimation of anthocyanins.
3.2.2.2. **Spectrophotometric estimation of carotenoids and chlorophyll**

For simultaneous analysis of both carotenoids and chlorophylls, total carotenoid content in the extract was estimated by spectrophotometric reading by using the following equation (Lichtenthaler 1987),

Chlorophyll a, \( Ca = 11.24 \ A_{661.5} - 2.04 \ A_{645} \),

Chlorophyll b, \( Cb = 20.13 \ A_{645} - 4.19A_{661.5} \),

Total carotenoids + xanthophyll, \( C + x = (1000 \ A_{470}) - [(1.9Ca) + (63.14Cb)] \ V \times DF / 214 \),

where \( V \) = Total volume of the extract, DF = Dilution factor.

3.2.2.3. **High performance liquid chromatography analysis of carotenoids**

HPLC system (LC-10A; Shimadzu, Kyoto, Japan), equipped with a Photo Diode Array detector (PDA, SPD-M 10 AVP) was used for analysis of major carotenoids. Injection volume of the sample was 20 µL and the mobile phase consisted of acetonitrile: methanol: ethyl acetate (80:10:10 v/v). Carotenoids were separated using a Phenomenex Gemini C18 reverse phase stainless steel column (250 × 4.6 mm) in isocratic mode with solvent flow rate of 1 mL/min. Each chromatogram was acquired at 450 nm by using the software LC solution (Shimadzu, Japan). Different carotenoids were identified by comparing the retention time of each peak with the peak obtained with respective standard, as well as comparing respective UV spectra and by spiking with their standards, followed by mass spectral analysis. Quantification of \( \beta \)-carotene and lutein was done by considering peak area and comparing to that of known concentration of respective standard.

3.2.3. **Determination of phenolics by HPLC**

Vacuum-dried samples (extractives) were re-dissolved in methanol and passed through Sep-Pack C18 cartridge. Each purified eluent was passed through membrane filters (0.4 µm pore size) before HPLC analysis. Separation of phenolic compounds was performed with a Phenomenex Gemini C18 reverse phase stainless steel column (250 × 4.6 mm) fitted to HPLC system (LC-10AT; Shimadzu, Kyoto, Japan) equipped with a PDA detector SPD-M 10 AVP, system controller CBM-20A and the data analyzed with
the software LC solution, Shimadzu, Japan. Isocratic elution was carried out using a mobile phase consisting of water: methanol: acetic acid (80:18:2 v/v) at a flow rate of 1 mL/min (Neo et al. 2010). Chromatograms were monitored at 280 nm and 320 nm. Chlorogenic acid and caffeic acid were detected at 320 nm. The injection volume for samples was 20 µL. Identification of phenolic acids was done by comparing retention time of each peak with that of respective standard. Calibration curves were made for each standard and the concentration of each phenolic compound was calculated by comparing respective peak areas. Chlorogenic acid and caffeic acid standards were dissolved in HPLC-grade methanol to obtain a final concentration of 1 mg/mL and appropriate dilutions were made with the same solvent. Further confirmation of phenolics was done by mass spectral analysis performed in a Waters Q-Tof Ultima Global (Manchester, UK) system equipped with the MassLynx 4.0 software and analyzed by the following parameters. ESI negative ion mode was used to form deprotonated ions and the optimum conditions for the analysis of these compounds were capillary voltage 3.5 KV, cone voltage 50 KV, source temperature 120 °C, desolvation temperature 300 °C, cone gas flow 50 L/h, desolvation gas 500 L/hr.

3.2.4. Determination of antioxidant activity

Extracts of total carotenoids and total phenolics of the coriander foliage, obtained before and after treatment, were evaluated for their antioxidant activities by analyzing superoxide scavenging activity and protection against hydroxyl radical induced DNA damage.

3.2.4.1. Superoxide radical scavenging activity of elicited sample

Superoxide scavenging activity of carotenoid and phenolic fraction was determined by the method of (Nishimiki et al.1972). Superoxide scavenging activity is inversely proportional to the decrease in absorbance and the percentage inhibition was determined by,

\[ \% \text{ Inhibition} = \frac{(A0 - A1)}{A0} \times 100 \]

where, A0 was the absorbance of the control reaction mixture (without extract) and A1 was the absorbance of the sample reaction mixture.
3.2.4.2. Hydroxyl radical scavenging activity

The extent of DNA damage caused by hydroxyl radical generated by Fenton's reagents (Fe$^{3+}$-ascorbate-EDTA-H$_2$O$_2$ system) according to Cao, Chen, Zheng, and Zheng (2008) with slight modification. The reaction mixture (1 mL) contained calf thymus DNA (0.8 mg/mL), Fe$^{3+}$chloride (1 mmol/L), EDTA (1.0 mmol/L), and H$_2$O$_2$ (1.0 mmol/L), with and without test extracts (phenolics and carotenoids) of varying concentrations in sodium phosphate (NaH$_2$PO$_4$-Na$_2$HPO$_4$) buffer (pH 7.4, 50 mmol/L). Ascorbic acid (10 mmol/L) was added to trigger the reaction by reducing Fe$^{3+}$ to Fe$^{2+}$, and the reaction mixture was incubated at 37 °C for 30 min. Fenton's assay reagents were prepared just prior to use. To 1 mL of the above mixture, 1 mL of TBA (0.5 g in 100 mL of 25 mmol/L NaOH) and 1 mL of TCA (10 g/100 mL aqueous solution) were added. The mixture was incubated in a boiling water bath at 80 °C for 60 min. After centrifugation at 7700 × g for 10 min, pink color formed was spectrophotometrically measured at 532 nm. Hydroxyl radical scavenging activity was calculated by the following equation:

\[
\% \text{ hydroxyl radical scavenging activity} = \left(1 - \frac{A_s}{A_c}\right) \times 100;
\]

where ‘As’ is the absorbance of the sample and ‘Ac’ is the absorbance of the control.

3.2.4.3. Determination of hydroxyl radical induced DNA damage by gel electrophoresis

The extent of DNA damage induced by hydroxyl radicals, and the ability of total carotenoid and phenolic extract to protect DNA, were studied by DNA nicking assay, as described by Lee, Kim, Kim and Jang (2002) with minor modifications. The reaction mixture contained 5 µg of calf thymus DNA in Fenton’s reagent (H$_2$O$_2$ 30 mmol/L, ascorbic acid 50 µmol/L, FeCl$_3$ 80 µmol/L) in Tris-EDTA buffer. The samples, dissolved in DMSO, were added in different concentrations, and the final reaction volume was made up to 20 µL. This mixture was incubated for 30 min at 37 °C and DNA was subjected to agarose-gel (1 g agarose in 100 mL Tris-acetate-EDTA buffer) electrophoresis, followed by ethidium bromide staining by following standard method. The gel was visualized and documented using a gel documentation system (Herolab GmbH Laborgeraete, Wiesloch, Germany).
3.2.5. Gene expression studies

3.2.5.1. RNA isolation

Total RNA from MeJa-treated and control foliage (100 mg) was extracted from samples collected at different periods after elicitor application in case of treated plants. RNA was isolated by using RNA isolation kit (Sigma-Aldrich) according to the manufactures instruction.

3.2.5.2. DNase treatment

To eliminate the contaminated DNA in the RNA extract, the latter was treated with DNase enzyme. To 20 µL of RNA, 2 µL of reaction buffer (10X) and 2 µL of DNase 1 were added. Mixed well and incubated for 15 min at room temperature. 1 µL of stop solution was added to inactivate the DNase 1. To denature both DNase 1 and RNA, the mixture was incubated at 70 °C for 10 minutes, followed by chilling on ice.

3.2.5.3. Characterization of isolated RNA by spectrophotometric method

After DNase treatment quantity of RNA was analysed by nanodrop spectrophotometer (Thermo scientific). Purity was determined from the absorbance ratio at 260 nm and 280 nm. Pure RNA exhibited a A260 / A280 ratio of 2. Integrity of RNA was checked by electrophoresis in formaldehyde denaturing gel stained with ethidium bromide.

3.2.5.4. RNA gel electrophoresis

To prepare 200 mL of molten agarose solution, 3 g of agarose was added to 170 mL of distilled water and melted in a microwave oven. When gel was cooled, 20mL of pre warmed 10X MOPS buffer and 10 mL of pre-warmed 37% formaldehyde were added and allowed to solidify at room temperature. RNA sample was prepared by adding 3 µL of formaldehyde loading dye (Fermentas), incubated at 65 °C for 15 min and chilled on ice. Solidified gel was transferred to a gel electrophoresis unit containing 1X MOPS buffer. Then denatured RNA samples were loaded and allowed to electrophorese for 45 min. The gel was stained with ethidium bromide dissolved in sterile water. Afterwards, the gel was documented by using gel documentation system (Herolab, Germany).
3.2.5.5. First strand cDNA synthesis

For the preparation of each cDNA, 1 µg of RNA template mixed with 1 µL oligo dT and 1 µL random hexamer and the volume was made up to 10 µL with DEPC water and incubated at 65 °C for 5 min, and quickly chilled. When chilling was complete, 8 µL total of (5X cDNA buffer 4 µL per reaction, dNTP mix 2 µL, Verso cDNA mix 1 µL and RT enhancer 1 µL) were added per reaction. Mixed gently and the reaction was allowed to occur at 25 °C for 5 min, 42 °C for 1 h, 95 °C for 2 min.

3.2.5.6. Semi quantitative PCR analysis

The gene-specific primers for ten genes were designed using the software of Integrated DNA Technologies Inc. USA. Reverse transcriptase PCR analysis was carried out using PCR mixture (20 µL) containing 1 µL of cDNA template prepared from 1 µg of RNA, 1X PCR buffer, 200 µmol/L dNTPs, 1 U of taq DNA polymerase and 0.1 µmol/L of both forward and reverse gene specific primer. PCR was performed at initial denaturation at 94 °C for 4 min, 30–35 cycles (30 s at 95 °C, annealing Tm for 45 s, extension at 72 °C for 1 min), final extension at 72 °C for 10 min using gradient PCR thermo-cycler (Palm-Cycler, Corbett Research, Sydney, Australia). Semi quantitative expression analysis of genes was done for primers that were used to amplify CsIPI, CsPSY, CsPDS, CsZDS, CsCRTISO, CsCHYB, CsCHYE, CsLYCB, CsLYCE, and CsCCD as listed in Table 3.1. A house keeping gene from coriander the 18s rRNA, was used as an internal standard. The difference in relative transcript level of each gene was normalized based on the amplification of 18s rRNA, with forward and reverse primers of the same. For calculating the transcript abundance of each gene after treatment, transcript levels of control plants (without treatment) of respective time equivalent to that of elicitor treatment were taken for comparison. The number of cycles for amplification of each gene was optimized for the uniform expression pattern. To characterize the expression level of ten carotenoid pathway genes in normal and elicited conditions, semi-quantitative reverse-PCR analysis was used. All treated and control cDNA were amplified with gene-specific primers in linear range of 29 to 35 cycles. Control RT-PCR with 18S rRNA primer amplified all cDNA in a uniform range showing the presence of equal amount of template used for each reaction. Identical
conditions were used for each PCR reaction. The transcript abundance was quantified by calculating the band intensity by using EASY WIN Software.

Table 3.1. Gene-specific primers, annealing temperatures, total number of amplification cycles and GenBank ID of primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence(5’-3’)</th>
<th>Annealing temperature (°C)</th>
<th>No of cycles</th>
<th>Genebank ID/Reference No</th>
<th>Ampli con size (bp)</th>
</tr>
</thead>
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<tr>
<td>IPP-forward</td>
<td>TCCGAGCTTATCGAGGAAAA</td>
<td>58</td>
<td>40</td>
<td>-</td>
<td>274</td>
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<td>IPP-reverse</td>
<td>TTCATGTTCCTCCCACTTGC</td>
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</table>

3.2.5.7. Quantitative Real Time polymerase chain reaction analysis of genes

To examine the expression of the coriander carotenoid biosynthetic genes, the cDNA samples were used as templates in real-time PCR assay in the presence of a Power SYBR Green PCR Master Mix Invitrogen, Life Technologies (Karlsruhe,
Germany) and carotenogenic gene-specific primers (Table 3.1). The reactions were performed in a Real-Time PCR: CFX96 Touch System with CFX Manager™ software (BioRad, Hercules, USA). Thermal cycling conditions consisted of a first step of 50 °C for 2 min, denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 1 min at 60 °C. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method as described by (Livak and Schmittgen 2001). All data were normalized first with the level of the 18s internal transcript control and then with the expression of the controls (relative expression). Values reported represent the averages of three independent trials with three repeats.

3.2.6. Ligation of PCR product to T-tailed vector

The A-tailed purified PCR product of isolated differential amplicons were cloned by ligating to pTZ57R/T cloning vector using T/A Clone PCR Product Cloning Kit (InsTAclone PCR Cloning Kit Thermo scientific) and transforming competent cells of Escherichia coli strain DH5α.

For each reaction, the following components were added in a thin-walled 0.2 mL PCR reaction tube: Plasmid vector pTZ57R/T (1.0 μL), PCR fragment (4.0 μL), 10X Ligase buffer (1.0 μL), T4 DNA ligase, 5 U/μL (0.5 μL) volume made up to 10 μL with deionized water. The reaction components were mixed by pipetting or by a brief spin. The reaction mixture was incubated at 16 °C for four h. The enzyme was inactivated by heating the reaction to 65 °C for 10 min.

3.2.6.1. Transformation of E. coli using the ligation reaction mix

3.2.6.1.1. Preparation of competent cells using CaCl₂

A single colony of E. coli (DH5α strain) from a plate, freshly grown for 15-20 h at 37 °C was picked and transferred into 50 mL of LB broth in a 250 mL conical flask. The culture was incubated at 37 °C with rigorous shaking. The OD$_{600}$ of the culture was determined periodically to monitor cell growth. When the OD$_{600}$ reached 0.4-0.5, the cells were transferred aseptically to 50 mL sterile polypropylene tube. The culture was cooled by storing the tube on ice for 10 min. The cells were recovered by centrifugation at 4000 rpm for 8 minutes at 4 °C. The media was decanted from the cell pellet. The cell pellet
was re-suspended in 10 mL of ice-cold 0.1 M CaCl₂ and stored on ice. The cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4 °C. The fluid from the cell pellet was decanted and the tubes were kept in an inverted position for 1 min to allow the last traces of fluid to drain away. The cell pellet was re-suspended in 10.0 mL of ice-cold 0.1 M CaCl₂ and cells were stored at 4 °C for 4-8 hours.

3.2.7. Transformation of competent cells

About 200 µl suspensions of competent cells were added to sterile micro-centrifuge tubes. Plasmid DNA (~50 ng) or 4 µL of ligation mixture was added to each tube. The contents of the tubes were mixed by swirling gently and the tubes were stored on ice for 30 min. Control samples were included as following: (a) competent cells that received supercoiled plasmid DNA and (b) competent cells that received no plasmid DNA. The tubes were transferred to water bath set at 42 °C for 90 s to subject the cells to heat shock. Then sudden cooling in ice where the cells were allowed to chill for 45 s. To each tube, 800 µL of SOC medium was added and the cultures were incubated for 45 min at 37 °C.

3.2.7.1. Selection of transformants/recombinants

About 100 µL of transformation mix was plated onto LB agar plates containing 100 µg mL⁻¹ ampicillin, 0.5 mM IPTG and 80 µg mL⁻¹ X-Gal. The plates were incubated overnight at 37 °C for allowing the colonies to grow.

3.2.8. Isolation of plasmid DNA from the transformed colonies

Single colonies of bacteria were inoculated in 2 mL of LB broth containing antibiotic and grown overnight in a shaker incubator at 37 °C and 180 rpm. 1.5 mL of the overnight culture was transferred to a 1.5 mL micro centrifuge tube and the cells were harvested by centrifugation at 10,000 rpm for 2 min. After discarding the supernatant, 100 µL of solution I was added and vortexed vigorously until no visible clumps of cells were observed. The samples were kept on ice for 5 min. About 200 µL of freshly prepared alkaline solution (solution II) was added to the tube and mixed gently by inverting the tubes several times until the lysed cell suspension became clear. The samples were kept on ice for 5 min, 150 µL of ice-cold potassium acetate solution (solution III) was added, and tubes were inverted gently. The tubes were centrifuged at
10,000 rpm for 10 min. The supernatant was transferred to a fresh tube and equal volume of phenol-chloroform was added and vortexed thoroughly. Centrifugation at 10,000 rpm for 10 min separated the two phases. The upper aqueous phase was transferred to a fresh tube and equal volume of chloroform was added. The tubes were centrifuged at 10,000 rpm for 10 min. The upper aqueous phase was transferred to a fresh tube and 2 volumes of absolute ethanol were added. The tubes were kept at -20 °C for 1 h and kept overnight at 8 °C for precipitation. The tubes were centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. The pellet was washed with 300 µL of 70% ethanol and the air-dried pellet was dissolved in 20 µL of TE buffer. Samples were tested by carrying out agarose gel (1%) electrophoresis along with control plasmid.

3.2.9. Sequencing and data alignment of the clones

DNA sequencing was carried out using M13F and M13R primers by dideoxy chain termination method. The reaction was carried out in an automatic DNA sequencer (ABI prism, Applied Biosystems, USA) with fluorescent dideoxy chain terminators at the author’s laboratory (Central Food technological Research Institute, Mysore). Chromatograms obtained were edited by the program ChromasLite. Data alignment was done by using BLAST program of National Centre for Biotechnology (NCBI), sequences were submitted to NCBI and accession numbers were obtained.

3.2.10. Studies using carotenoid-pathway inhibitors

Fosmidomycin (an inhibitor of MEP pathway), norflurazon (NF), a phytoene desaturase inhibitor (Sandmann and Albrecht 1990; Steiger et al. 1999) and amitrol (known to inhibit lycopene cyclase) were used to strategize additional experimental conditions for studying the regulation of genes involved in carotenoid biosynthesis under normal and elicited conditions. Aliquots were taken from the stock solutions in order to obtain the active concentration for each inhibitor. Preliminary investigations were carried-out in order to determine the optimal concentration of each inhibitor. The following concentrations of fosmidomycin dissolved in water to obtain a final concentration of 50, 100, 150 and 200 µmol/L were screened as foliar spray. Since concentrations below 150 µmol/L of fosmidomycin had no influence on the
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biosynthesis of β-carotene, 200 µmol/L fosmidomycin was selected for the present study.

Coriander seeds were germinated and grown at 25 or 30 °C in a growth chamber with a 12 h photoperiod and relative humidity of 80% on soil moistened with tap water. Coriander plants were treated with norflurazon (100 µmol/L) and amitrol (200 µmol/L) by foliar application. For this, leaves at profuse growth stage were selected. Control plants were sprayed with water and grown under same conditions. After foliar spray with each inhibitor, 0.1 g of leaf sample was collected for simultaneous gene expression analysis and major carotenogenic genes expression were quantified. Quantification of each carotenoid was done by HPLC method at different periods in both control and treated samples.

3.2.11. Statistical analysis

Each experiment was repeated three times with at least three replicates. All the calculations were done separately for each experiment. Statistical analysis of all the data was done using SPSS 17 software (Chicago, IL, USA). Data presented are mean of triplicate values with their standard deviations, and significant difference of the treatments were analyzed by comparing with the control by one way ANOVA followed by Dunnet’s test (post-hoc).

3.3. Results

3.3.1. Effects of methyl jasmonate and salicylic acid treatments on carotenoids and chlorophylls in cv. GS4 Multicut

Enhanced carotenoids and chlorophyll content after elicitor treatment was quantified by HPLC method as in Figure 3.2. The enhancement pattern of total carotenoids, β-carotene, lutein, chlorophylls, total phenolics and chlorogenic acid at different time periods after the plant growth regulator-treatment was similar in both cv. GS4 Multicut and cv. Mahak, even though the extent of response varied from each other. GS4 Multicut, the high pigment and biomass yielding coriander cultivar, showed better response to all the treatments than that of Mahak. In cv. GS4 Multicut, foliar treatment with MeJa (10 µmol/L) was found most suitable for the enhancement of
carotenoids, where three days after treatment, 6.8, 3.9, 6.1, 3.3 and 2.4 fold increase was observed for total carotenoids, β-carotene, lutein chlorophyll a and chlorophyll b respectively.

A previous study in apple (Pérez et al. 1993) recorded a threefold increase in β-carotene after MeJa treatment, although the levels of chlorophyll a and lutein were found to decrease. Application of MeJa was found to enhance the accumulation of carotenoids in tomato (Saniewski and Czapski 1983). In the present study, considerable decrease in chlorophyll a and chlorophyll b was observed only after 7th and 12th days after treatment with MeJa. In another study with lettuce, MeJa was found to suppress the formation of total carotenoids, although phenolics were enhanced (Kim et al. 2007). When HPLC profiles of carotenoids (Figure 3.2) and chlorophylls after treatment were analyzed quantitatively, significant increase in their accumulation was noted, which is evident from the x-axis scale. Foliar treatment with SA (500 µmol/L) resulted in 5.4 fold increase in total carotenoids after three days. The increase in β-carotene (2.6 fold), lutein (4.6 fold), chlorophyll a (2.8 fold) and chlorophyll b (1.8 fold) when analyzed with HPLC/MS was also found to be significant.

In coriander, chloroplast-type carotenoids are represented by lutein, violaxanthin, and β-carotene, which are essential for photosynthesis and lutein, the most abundant carotenoid in the leaves.
Figure 3.2. HPLC chromatograms of carotenoids in coriander foliage, before and after elicitation with methyl jasmonate (10 µmol/L), where chromatograms were recorded at 450 nm
Lycopene is not detected in our HPLC analysis, may be the complete utilization of this substrate for the synthesis of downstream carotenoid products. β- carotene level reaches its maximum on 3rd day and showed a decreasing trend afterwards. Lutein on the other hand also reaches highest on 3rd day, but there was no reduction observed after this time and is consistent even after 12 days of treatment.

In case of treatment with 10 μmol/L of MeJa, after an increase on first day, sudden decrease in carotenoids were observed on second day Figure 3.3.B. However, after two days carotenoids reached a maximum level, especially the lutein content increased. Other carotenoids were also found to increase at the same time. After three days all carotenoids decreased in a uniform manner.

Treatment with 100 μmol/L MeJa also enhanced carotenoids in which, at the initial time periods, both β-carotene and lutein concentration were similar with high other carotenoids Figure 3.3.C. On the third day of treatment, lutein was enhanced, which was found to suddenly decrease later and this trend was also found in the case of β-carotene.

Contrary to 10 and 100 μmol/L MeJa, 500 μmol/L of elicitor enhanced β-carotene lutein and other carotenoids from the day one and maximum on third day, with a further uniform or slight decrease in content β-carotene and lutein were observed Figure 3.3.D. Even though, total carotenoids and lutein increased in the same pattern compared with control plants, higher concentration (1000 μmol/L MeJa) decreased all carotenoids compared with other low concentration of the same elicitor Figure 3.3.E. For both 500 and 1000 μmol/L MeJa lutein concentration was not decreasing, where as other carotenoids showed a descending trend after third day.

The other plant growth regulator used was salicylic acid, which also enhanced carotenoids after three days and uniform concentration of both lutein and β-carotene were obtained for 10 μmol/L. Whereas, for 100 μmol/L an irregular pattern in concentration were obtained with other carotenoids also. Higher concentration of SA showed same pattern of lutein and β-carotene elicitation, as noted for treatment with MeJa, with maximum accumulation on the third day and decreasing later.
Figure 3.3. Changes in profiles (g/kg DW) of major carotenoids in coriander foliage (cv. GS 4 Multicut) after treating with elicitors - methyl jasmonate and salicylic acid. Significance of the treatment was tested by comparing with the respective control values using Dunnett’s test. A. Control. B. Meja 10 μmol/L; C. Meja 100 μmol/L; D. Meja 500 μmol/L; E. Meja 1000 μmol/L; F. SA 10 μmol/L; G. SA 100 μmol/L; H. SA 500 μmol/L; I. SA 1000 μmol/L
The observation made in this study that higher SA concentrations enhanced the accumulation of total carotenoids and xanthophylls is supported by similar results in wheat and moong seedlings (Moharekar et al. 2003), and in salt stressed maize plants (Khodary 2004). Since SA is known to affect the photosynthetic rate in plants, its direct role in the enhancement of carotenoids may be expected since carotenoids are needed to quench several species of pro-oxidants formed continuously during photo-oxidation. However, the application of MeJa to mature foliage (60–70 days old plant) showed no significant increase in total carotenoids and chlorophylls, particularly of chlorophyll a and lutein compared with control (data not given). The highest concentration of MeJa (1000 µmol/L) not just suppressed carotenoids and chlorophylls, but promoted the senescence of leaves after seven days of treatment. Contrarily, such high levels of SA had no adverse effects, either on the concentration of carotenoids or on the senescence of leaves.

In control plants, chlorophyll a and b contents were found to increase from the day one to twelfth day. After the MeJa treatment, chlorophylls also increased along with carotenoids on the same day (3rd) and decreased afterwards (Figure 3.4.B). But for treatment with SA at 100 µmol/L, both chlorophyll a and chlorophyll b were found to increase from day 1 to 12 days Figure 3.4. As the MeJa concentration increased, the chlorophyll content decreased, whereas, SA applied at higher concentration (both 500 and 1000 µmol/L) increased chlorophyll content Figure 3.4. H.
Figure 3.4. Changes in profiles (g/kg DW) of chlorophyll a and chlorophyll b in coriander foliage (cv. GS4 Multicut) after treating with elicitors - methyl jasmonate (MeJa) and salicylic acid (SA). Values are represented as mean ± SD of three replicates. A. Control; B. MeJa 10 μmol/L; C. MeJa 100 μmol/L; D. MeJa 500 μmol/L; MeJa 1000 μmol/L; F. SA 10 μmol/L; G. SA 100 μmol/L; H. SA 500 μmol/L; I. SA 1000 μmol/L
3.3.2. Effects of methyl jasmonate and salicylic acid treatments on carotenoids and chlorophylls in cv. Mahak

In cv. Mahak (the low carotenoid-yielding type), the pattern of accumulation of carotenoids was similar. But the concentration was much lower. Figure 3.5 showed the pattern of carotenoids β-carotene and lutein accumulation in Mahak after foliar treatment with MeJa and SA. Even though 10 μmol/L MeJa enhanced β-carotenes and lutein, higher concentrations of 100 μmol/L and 500 μmol/L enhanced more of lutein than β-carotene (Figure 3.5.C & D). Treatment with SA also enhanced carotenoid to the same extent as that for MeJA, after two days. But it enhanced other carotenoids also. In this cultivar also, elicitor application enhanced both types of chlorophylls. As in cv. GS4, in Mahak also the chlorophyll a was enhanced after two days, with a decreasing trend then onwards (Figure 3.6). But in cv. Mahak, both chlorophyll a and b were not found to decrease after treatment with MeJa. For SA treatment, 100 μmol/L effected higher increase of chlorophyll a than 10 μmol/L. SA concentrations ranging from 500 to 1000 μmol/L suppressed both chlorophyll a and chlorophyll b (Figure 3.6.G & H).
Figure 3.5. Changes in profiles (g/kg DW) of major carotenoids, β-carotene and lutein in coriander foliage (cv. Mahak) after treating with elicitors - methyl jasmonate and salicylic acid. Values are represented as mean ± SD of three replicates. A. Control; B. Meja 10 µmol/L; C. Meja 100 µmol/L; D. Meja 500 µmol/L; Meja 1000 µmol/L; F. SA 10 µmol/L; G. SA 100 µmol/L; SA 500 µmol/L; SA 1000 µmol/L
Figure 3.6. Changes in profiles (g/kg DW) of chlorophyll a and chlorophyll b in coriander foliage (cv. Mahak) after treating with elicitors - methyl jasmonate (MeJa) and salicylic acid (SA). Values are represented as mean ± SD of three replicates. A. Control; B. Meja 10 μM; C. Meja 100 μM; D. Meja 500 μM; E. Meja 1000 μM. F. SA 10 μM; G. SA 100 μM; H. SA 500 μM; I. SA 1000 μM
3.3.3. Phenolic compounds

Application of SA (10 μmol/L) enhanced the content of total phenolics up to 30.4 g/kg DW from only 7.3 g/kg DW found in control after 24 h of treatment. This level of phenolics in control plants is similar to an earlier study in coriander that recorded total phenolics as 9 g/kg DW gallic acid equivalent (Shan et al. 2007). Although (Wong and Kitts 2006) reported 1.1 g caffeic acid/kg (FW) in coriander foliage, HPLC analysis of methanolic extract in the present study showed chlorogenic acid (a hydroxycinnamic acid derivative) as the major compound, followed by very low level of caffeic acid (Figure 3.7.A). Variations in the profile of phenolics and their concentrations may be due to differences in extraction solvents and methods, since solvents display a wide variation in polarity. In the current study, the level of chlorogenic acid was found to increase by threefold after the treatment with SA (10 μmol/L), which was neither drastically affected by higher levels nor inhibited at late treatment period (12th day).

In another study with purple cornflower (Echinacea purpurea L. Moench.), foliar application of either MeJa or SA was observed to produce tenfold higher phenolic compounds than control plants (Kuzel et al. 2009). Chemicals, particularly the foliar application of 0.2 mol/L calcium chloride, increased the content of chlorogenic acid and total phenolic compounds in lettuce plant (Perucka and Olszówka 2011). Thus, between the two plant growth regulators, MeJa at 10 μmol/L concentration was found more suitable for the enhancement of micronutrients and nutraceutically important compounds in coriander. However, for similar extent of enhancement, a much higher level (500 μmol/L) of SA was required, although this plant growth regulator could also impart significant beneficial effects. The peaks observed in HPLC analysis were further characterized by comparing their respective UV spectra, mass spectra and retention time with those of standards. In ESI negative mode, deprotonated ion [M–H]⁻ at m/z 353 (Figure 3.7.F) with λmax 327 (Figure 3.7.G) was found similar to that of chlorogenic acid standard. Caffeic acid showed λmax 322 (Figure. 4E) and [M–H]⁻ at m/z 179. Phenolic compounds increased and attained a maximum level immediate, i.e., within the first day of treatment where the increase in total phenolics and chlorogenic acid for MeJa (10 μmol/L) treatment was 3 and 5 fold respectively.
Figure 3.7. HPLC chromatograms of phenolic extracts of coriander foliage, before (A) and after (B) elicitation with salicylic acid (10 µmol/L), and chlorogenic acid standard (C), UV spectrum of chlorogenic acid (D) and UV spectrum of caffeic acid (E). Chromatograms were recorded at 320 nm. (F) represents mass spectrum of chlorogenic acid and G represents mass spectrum of caffeic acid.

Figure 3.8 represents the total phenolics and chlorogenic acid cv. GS 4 (A & B) and cv. Mahak (C & D). While there was enhancement of chlorogenic acid even with 10 µmol/L of MeJa, there was a trend of negative effects with the increase in this treatment concentration. Since it is established that chlorogenic acid helps lowering the peroxidation of low-density lipids and reduce total cholesterol formation, its importance in reducing the risk of cardiovascular diseases in humans (Nardini et al. 1995) is worth considering. For these reasons, attempts have been made to engineer plants producing high chlorogenic acid and proven successful in some food crops (Niggeweg et al. 2004). The increase in chlorogenic acid content may occur through the elicitation of phenyl propanoid pathway, which is chiefly responsible for the induction of defense-related compounds.
Figure 3.8. Changes in profiles (g/kg DW) of total phenolics (A) and chlorogenic acid (B) in cv. GS 4-Multicut and cv. Mahak (C) and (D) after treatment with elicitors - methyl jasmonate and salicylic acid. Values are represented as mean ± SD of three replicates
3.3.4. Antioxidant activity of carotenoids and phenolics after elicitation

3.3.4.1. Superoxide scavenging activity

In cell biology, it is well understood that superoxide radicals are generated because of various biological reactions. If not nullified immediately by various mechanisms that a normal cell equipped is with these radicals would cause cellular toxicities, degrade nucleic acids, and inactivate several enzymes, cause alterations in the integrity of cell membranes, leading to lysis and death of the cell. Since superoxide radical anions are the precursors of hydroxyl radicals, which are highly reactive, the superoxide radical scavenging ability of coriander extracts was measured. The scavenging ability (IC\textsubscript{50} value) of carotenoids fraction of control was 14.2 mg/L and after the treatment was 5.01 mg/L, the latter being much higher than that of standard β-carotene (IC\textsubscript{50} 27.33 mg/L) (Figure 3.9.A). Similar efficacies of phenolic extracts are shown in (Figure 3.9.B), where the IC\textsubscript{50} value for total phenolics of control foliage was 56.1 mg/L whereas after the treatment, the IC\textsubscript{50} of the extract showed higher efficacy of 45.5 mg/L, and that of standard chlorogenic acid was 36.9 mg/L. Even though chlorogenic acid is the major compound among phenolics, other unidentified compounds could also account for the difference in anti-oxidant activity.

3.3.4.2. Hydroxyl radical scavenging activity assay

While both carotenoids and methanolic extracts inhibited hydroxyl radicals in a concentration dependent manner, their higher bio-efficacies evident from treated samples (Figure 3.9.C). The scavenging efficacy of hydroxyl radicals was better in carotenoid extract obtained from treated foliage (with an IC\textsubscript{50} of 2.6 mg/L) than that from control foliage (IC\textsubscript{50} of 16.5 mg/L). This efficacy of coriander carotenoids is attributable to the high content of lutein, which contains two hydroxyl groups to scavenge hydroxyl radicals effectively, explaining the reason for lower efficacy of standard β-carotene (IC\textsubscript{50} 21 mg/L). Phenolic compounds, also known to be good scavengers of hydroxyl radicals, showed an IC\textsubscript{50} of 39.7 mg/L in control and 18 mg/L in treated sample, compared with IC\textsubscript{50} 30 mg/L of quercetin standard (Figure 3.9.D). These observations made on the improvement in antioxidant property further support the positive effects imparted by MeJa and SA.
Figure 3.9. Superoxide and hydroxyl radical scavenging activities of total carotenoids (A & C) and total phenolics (B & D) isolated from foliage of coriander cv. GS4 Multicut obtained from control and elicitor treated plants as compared with respective standards \( \beta \)-carotene, chlorogenic acid and quercetin. Values are represented as mean ± SD of three replicates. Significance of the treatment was tested by comparing with the control values using Dunnett’s test. \* \( p < 0.05 \), \** \( p < 0.01 \), \*** \( p < 0.001 \)

3.3.4.3. Hydroxyl radical induced DNA damage assay

Antioxidants ingested through food are known to scavenge free radicals, thereby preventing the damaging effects of free radicals on DNA, proteins, lipids and other cellular components. Antioxidants, which neutralize the deleterious effects of reactive oxygen species (ROS), are needed to protect cells by supporting them to effectively maintain a balance between ROS and antioxidants. Although certain micronutrients
such as ascorbic acid, β-carotene and several other carotenoids function as antioxidants, these molecules need to be spared for other nutritional functions. For instance, β-carotene is needed for the synthesis of vitamin A (retinol), which is not only involved in maintaining normal vision but also in cellular differentiation, normal reproduction, for healthy skin and hair, and various other functions. Therefore, simultaneous enhancement of several types of antioxidant molecules would be a preferred strategy, as demonstrated in the present study, for enhancing the health benefits of green foliar vegetables. Both carotenoids and phenolic compounds are known to compete with DNA for hydroxyl radicals and diminish thiobarbituric acid reacting substance formation, where the total antioxidant activity depends on the structure of the antioxidant molecule. Accordingly, as indicated above, the two hydroxyl groups present on terminal ends of lutein molecule, as well as its polar nature makes this compound an efficient antioxidant. Protective activity of phenolic compounds against hydroxyl radical induced damage was demonstrated by measuring the quantum of hydroxyl radical generated by Fenton’s reagents. Carotenoids (5 to 100 µg/mL) and phenolic compounds (5 to 100 µg/mL), when incubated with calf thymus DNA, showed that all extracts and standards (β-carotene and chlorogenic acid) were very effective in protecting DNA against hydroxyl radical induced damage (Figure 3.10.). In both types of extracts, lower concentrations were found more effective in scavenging hydroxyl radicals, as also observed in other studies (Nakayama, 1994).
Figure 3.10. Protection against hydroxyl radical induced DNA damage of carotenoids and phenolic extract of coriander foliage. (A) Lane 1: Control DNA; Lane 2: DNA + Fenton reagent (FR); Lane 3: β-carotene standard (10 µg/mL) + DNA + FR; Lane 4: carotenoid extract (5 µg/mL) + FR + DNA; Lane 5: carotenoid extract (10 µg/mL) + FR + DNA; Lane 6: carotenoid extract (15 µg/mL) + FR + DNA; Lane 7: carotenoid extract (20 µg/mL) + FR + DNA; Lane 8: carotenoid extract (50 µg/mL) + FR + DNA; Lane 9: carotenoid extract (100 µg/mL) + FR + DNA; Lane 10: chlorogenic acid standard (5 µg/mL) + FR + DNA; Lane 11: phenolic extract (10 µg/mL) + FR + DNA; Lane 12: phenolic extract (20 µg/mL) FR + DNA; Lane 13: phenolic extract (50 µg/mL) + FR + DNA; Lane 14: phenolic extract (100 µg/mL) + FR + DNA. Quantitation of relative DNA fragmentation after the treatment of calf thymus DNA with carotenoid (B) and phenolic (C) extracts at various concentrations is also shown.
3.3.5. Gene expression analysis

To check the integrity of RNA, all isolated RNA loaded in to agarose gel. All bands were intact. For all RNA, distinct 28S, 18S ribosomal RNA bands were found after ethidium bromide staining containing 1.5% denaturing gel. Bands were resolved in denaturing gel Figure 3.11.

Figure 3.11. Formaldehyde gel showing the integrity of isolated RNA

3.3.5.1. Carotenoid biosynthetic pathway genes expression in normal conditions

Carotenoids essential for photosynthesis, such as lutein, violaxanthin, and β-carotene, are generally abundant in leaves and due to their localization in chloroplasts, these are often nominated as ‘chloroplast-type carotenoids - among these, lutein dominates in green leafy vegetables. In the present study with coriander leaves, expression levels of genes coding for enzymes involved in isoprenoid and carotenoid biosyntheses were tracked, isolated and sequenced. Results that show the levels of expressions of carotenoid genes of coriander in normal conditions are represented in Figure 3.12. On the basis of the gene sequences, gene-specific primers were designed and the carotenogenic gene expressions were quantified by qRT-PCR using the primers. The transcript levels of most genes in coriander were similar. The enzymes catalyzing the desaturation are functionally diverse, of which two are found in plants. The enzyme phytoene desaturase (PDS) catalyzes the conversion of phytoene to ζ-carotene by means of phytofluene, whereas the conversion of ζ-carotene to lycopene via neurosporene is performed by ζ-carotene desaturase. Figure 3.13 shows the amino acid sequence similarity of the enzyme PDS from carrot and coriander. Cyclization of lycopene represents a central branch point in the carotenoid biosynthesis pathway, one route leading to the β,β-xanthophylls, zeaxanthin, violaxanthin and neoxanthin, and the
other leading to the β,ε-xanthophyll, lutein. The relative activities of ε-cyclase versus β-cyclase may determine the flow of carotenoids from lycopene to either α-carotene or β-carotene. The regulation of the two types of lycopene cyclizations could therefore be a major mechanism that controls carotenoid composition in vivo. Since ε-cyclization is the initial process in α-carotene biosynthesis, it may serve as the key regulator step in the pathway since its product δ-carotene leads to the α-carotene branch. The leaves of most plants show similar carotenoid profiles, representing both ε-, and β-carotenoids/β,β-carotenoids. In coriander, transcript levels of genes encoding for the rate-limiting enzymes involved in isoprenoid and carotenoid biosynthesis, such as PSY, PDS, ZDS and other important genes, LCYB, LCYE and CHXB remained high from day one to twelfth day, whereas, IPI transcript levels were low under normal conditions. The most prominent feature was the difference in the expression of LCYB and that of LCYE. Since the substrates and products of the ε- and β-cyclase enzymes are hydrophobic and are localized within the membranes of plant the enzymes are also tightly associated with plastid membranes (Camara and Dogbo 1986), whereas, carotenoid dioxygenases (CCDs) are located in cytosol (Rubio et al. 2008).

In plants, the allocation of lycopene substrate into pathways that lead to β-, ε-carotenoids (e.g. the abundant lutein) and to β, β-carotenoids (e.g. β-carotene, zeaxanthin, and violaxanthin) could be determined by the relative activities of the ε- and β-cyclase enzymes. According to HPLC quantification, in coriander leaves, the content of lutein was more than that of β-carotene before elicitor treatment as found in other GLVs. This may be because of the over expression of LCYE than that of LCYB. For example, in chrysanthemums, the difference in carotenoid components between petals and leaves occurred due to differences in the expression levels of LCYB and LCYE (Kishimoto and Ohmiya 2006). In addition, the repression of LCYE expression increases the β,β-carotenoid content in potato tubers and B. napus seeds (Diretto et al. 2006; Yu et al. 2008).
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Figure 3.12. Expression patterns of carotenoid pathway genes in coriander leaves (normal conditions) at different time intervals. *IPI*, isopentanyl isomerase; *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *ZDS*, zeta carotene desaturase; *CRTISO*, carotene isomerase; *LCYB*, lycopene β-cyclase, *LCYE*, lycopene ε-cyclase; *CHXB*, carotene β-hydroxylase; *CHXE*, carotene ε-hydroxylase

Figure 3.13. Amino acid sequence alignment of phytoene desaturase (PDS) from coriander and carrot. Highlighted regions (bracketed) show the conserved amino
acids. Asterisks indicate completely identical amino acid residues. The protein sequences were aligned using the program ClustalW (http://embnet.vital-it.ch/software/ClustalW.html)

Certain other studies showed that targeting the branch point either positively (LCYB enhancement) or negatively (LCYE inhibition) did not eliminate lutein accumulation and enhancing LCYB increased the accumulation of lutein along with β-carotene (Diretto et al. 2006). This indicates that LCYE is not a limiting step and that the α-branch benefits from the increased pathway flux, because the α-branch also requires LCYB activity.

3.3.5.2. Gene expression analysis of carotenoid biosynthetic genes after treatment with methyl jasmonate

Knowledge of the expression profile of genes involved in the carotenoid pathway (metabolic and catabolic pathways) is essential, as it can provide information about the regulation of these pathways over time, when correlated with carotenoid accumulation. Starting from the pathway, among the carotenogenic genes, the expression of PSY was evaluated as it encodes the first enzyme in the carotenoid biosynthetic pathway, as well as the expression of other important upstream genes like PDS, ZDS, CRTISO. Other key genes which determine the concentration of β-carotenoids and xanthophylls such as enzymes in the β,ε branch and enzyme in the β,β branch. Carotenoid catabolic enzymes are also very important, since the breakdown products in turn regulate the pathway genes (Walter and Strack 2011). Therefore, experiments were targeted to obtain information regarding not only the overall flux of the carotenoid pathway but also the regulation of the flux direction to each branch from the pathway. In the present study, after treatment with MeJa, the transcriptional expression profiling of the carotenoid genes was monitored simultaneously by qRT-PCR method. To obtain information about the regulatory mechanisms involved in carotenoid biosynthesis, the expression levels of genes involved in the metabolic and catabolic pathways of carotenoids were evaluated in high carotenoids producing cultivar GS4 Multicut and compared with the levels of specific carotenoids and the carotenoid concentration after treatment with inhibitors that affect target regulatory points in the pathway.
Isopentanyl pyrophosphate isomerase (*IPI*)

Carotenoid biosynthesis also depends upon the availability of isoprenoid substrates, and there is substantial evidence indicating the existence of a metabolic feedback mechanism that modulates the overall pathway. For *CsIPI* (Figure 3.14.A) an initial increase and the maximum transcriptional level occurred on day 3 and day 7 in the MeJa 10 µmol/L, and declined on day 12. However, for 100 µmol/L, there was a consistent level of expression after the treatment. Whereas, the highest transcriptional level of *IPI* in the Meja 500 µmol/L treatment occurred on day 1 and on 3rd day 6-fold higher level was observed compared to the control, with a decline sharply by 12th day.

Phytoene synthase (*PSY*)

The foremost key step in plant carotenoid biosynthesis is the synthesis of phytoene from GGPP. This reaction is catalyzed by PSY, which is probably the best studied enzyme of the plant carotenoid family. The PSY enzyme catalyzes a two-step reaction (Dogbo et al. 1988): the head-to-head condensation of two molecules of GGPP to form the reaction intermediate pre-phytoene diphosphate followed by the elimination of the diphosphate group from this intermediate in a complex rearrangement that involves a carbocation neutralization to form phytoene. Plant PSY enzymes typically use *all-trans* GGPP as a substrate to synthesize 15-cisphytoene, the isomer normally found in living cells. The biochemical properties of PSY enzymes from different plant tissues have been reviewed (Fraser and Bramley 2004). In Arabidopsis, only one PSY gene (*At5g17230*) has been reported, while a small gene family appears to encode PSY in most plants. For example, two genes encode PSY in tobacco and three genes in tomato, cassava, rice, and maize (Fraser et al. 1999; Busch et al. 2002; Giorio et al. 2008; Li et al. 2008; Welsch et al. 2008; Arango et al. 2010). Some isoforms are known to be involved in the biosynthesis of carotenoids in chloroplast-containing photosynthetic tissues such as leaves, whereas others participate in the production of carotenoids in chromoplasts (non-photosynthetic tissues) of the fruit (tomato PSY1), the seed endosperm (maize PSY1) or the root (tomato, maize and rice PSY3). The Arabidopsis *PSY* gene is expressed in all tissues, including both photosynthetic and
non-photosynthetic (Welsch et al. 2003), and it shows high rates of co-expression with the rest of the genes involved in the carotenoid pathway (Meier et al. 2011).

In coriander the initial increase and the maximum transcriptional expression of CsPSY (Figure 3.14.B) as a response to the MeJa (10 µmol/L) treatment occurred on day 3, and 7, where the transcript levels were 4- and 4.5-fold higher than that of control, respectively and after day 7 the levels showed descending pattern.

**Phytoene desaturase (PDS)**

On 500 µmol/L of methyl jasmonate 3.34-fold higher expression of PDS was observed on day 1 itself. Among three MeJa treatment groups, for 10 µmol/L, the first peak of transcriptional level of PDS was 11.7-fold higher (Figure 3.14.C) occurred on day 3, whereas the highest PDS steady-state mRNA transcriptional level occurred on day 7 (12-fold) in the low MeJa (10 µmol/L) treatment, and on day 2 in higher levels of MeJa (100 and 500 µmol/L) 10.5-fold and 10.7-fold higher respectively than the control. After these timespans, a similar decreasing trend in the transcriptional level was observed in both treatment groups.

**ζ-carotene desaturase (ZDS)**

The initial increased expression of CsZDS was 5.9- and 6.9-fold of controls on day 2 in both (100 µmol/L & 500 µmol/L) treatment, and for 10 µmol/L level of elicitor (MeJa) the content declined suddenly on day 2 after an initial accumulation as in (Figure 3.14.D). Two and one over-expression peaks were observed in MeJa 500 µmol/L and MeJa 100 treatments, respectively. For 10 µmol/L elicitor, the two peaks appeared on day 1 and 3 with 9.5- and 7.8-fold of transcriptional expression of control in treatment, respectively.
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Figure 3.14. Expression of carotenoid biosynthetic genes in C. sativum after foliar application with methyl jasmonate (10, 100 and 500 μmol/L). RT-PCR was performed as described in the Materials and methods. The PCR products were analysed by agarose gel electrophoresis.

Carotenoid isomerase (CRTISO)

CRTISO, which catalyses cis–trans reactions to isomerase the four cis-bonds introduced by the desaturases. In some plants, it has emerged as a regulatory node in the pathway (Isaacson et al. 2004) result in accumulation of cis-carotenes, such as 7,7-tetra-cis-lycopene, in the etioplasts (dark-grown plastids) of seedlings and chromoplasts of fruit. Cloning of tangerine from tomato revealed the presence of a carotenoid isomerase which was essential for the production of β-carotene and xanthophylls in plants.

The patterns observed for CRTISO (Figure 3.14.E) in 10 μmol/L were similar to those observed for CsPDS. Compared with control, it showed enhanced expression for
all the three concentrations of the elicitor. The initial increased expression of 3.5-fold higher than that of the control on day 1 in 500 µmol/L treatments.

**Lycopene cyclases (LCY)**

Carotenoid biosynthesis bifurcates after lycopene to produce ε- and β-carotenoids by enzymatic activity of two lycopene cyclases, *LCYE* that introduces ε-rings and β-LCY which introduces β-rings (Figure 3.15). Interestingly, the *LCYB* can catalyse the introduction of two β-rings into lycopene to form β-carotene via γ-carotene but the *LCYE* can only incorporate one ε-ring forming δ-carotene. For the formation of α-carotene both LCY-ε and β is essential to function. Regulation by *LCYE* is the first committed step coordinating carotenoid flux through the β-ε branch and modulates the ratio of the most abundant carotenoid, lutein to the β-carotenoids leading to changes in lutein content (Harjes et al. 2008; Cazzonelli et al. 2009; Howitt et al. 2009). A molecular synergism between *LCYE* and *LCYB* activities is an overall major determinant of flux through the branch leading to production of lutein, β-carotene and other xanthophylls cycle (XC) carotenoids (Yu et al. 2008). The xanthophyll carotenoids are major carotenoid sink metabolites accumulating in plant leaf tissues produced by the oxidative balancing of either α-carotene (lutein) or β-carotenes (zeaxanthin, violaxanthin and neoxanthin). Modulation of xanthophyll composition can greatly affect photosystem assembly, light harvesting, photoprotection and plants response to stress related signaling mechanism.

**Lycopene β-cyclase (LCYB)**

*LCY*-β genes have been identified from Arabidopsis (Cunningham et al. 1996) daffodil (Ac no. X98796), tobacco (Ac no. F13561) and pepper (Hugueney et al. 1995) MeJa treatment seemed to impart a moderate up-regulation in the expression of *CsLCYB* (Figure 3.15.F) than the other genes. The *CsLCYB* reached its highest transcriptional expression on day 1 in 10 µmol/L of MeJa treatment, whereas the expressions were only 1.8 and 2.5-fold higher than that of the control in the Meja 100 µmol/L and Meja 500 µmol/L treatments, respectively.
Lycopene ε-cyclase (*LCYE*)

This enzyme *CsLYCE* is thought to play an important role for the lutein formation, showed highest transcript level (four fold) at 7th day. For all the treatments, the gene expression was similar at different times.

Higher concentration of MeJa (500 µmol/L) have an immediate effect to which most of the genes expressed on day 1 with highest transcript level occurred on 2nd day and declined thereafter (Figure 3.15.G).

**Formation of xanthophylls:**

**Carotene hydroxylases**

In higher plants, xanthophylls are enzymically formed oxidation products of α and β-carotene. Hydroxylation of the C-3 and C-30 positions of α-carotene and β-carotene result in the formation of lutein and zeaxanthin via α-cryptoxanthin and β-cryptoxanthin, respectively. The introduction of hydroxyl moieties into β-ring carotenes is catalysed by hydroxylases. In Arabidopsis two β-carotene hydroxylases have been identified (Lange & Ghassemian 2003) and homologs have also been found in tomato and pepper (Bouvier et al. 1998).
Figure 3.15. Expression analysis of downstream genes in the pathway. *LCYB*. Lycopene β-cyclase; *LCYE*. Lycopene ε-cyclase; *CHXB*. β-ring carotene hydroxylase; *CHXE*. ε-ring carotene hydroxylase
β- hydroxylases (CHXB)

Irregular increasing trend in the expression of CsCHXB was observed, which doubled on day 1, followed by a sudden decrease, and on day 7 reached its peak as a response to treatment with MeJa (10 µmol/L). For CsCHXB 10 µmol/L two fold up regulation occurred, except for a slight decrease in its expression after 1 day. In 100 and 500 µmol/L, the expression showed a descending trend after 1 day (Figure 3.15.H).

ε- Hydroxylases (CHXE)

In case of 500 µmol/L MeJa treatment, a descending trend was observed after 3rd day for the expression of CsCHXE gene, which regulates the expression of ε-branch of pathway (Figure 3.15.I). Thus, CsLCYE may play an important role for the lutein formation, showed highest transcript level (three fold) at 7th day. For all the treatments, same genes were expresssive at different time points.

3.3.5.3. Carotenoid dioxygenases (CCDs)

Carotenoids are metabolized to apocarotenoids through the pathway catalyzed by carotenoid cleavage dioxygenases CCD1 catalyzes symmetrical 9–10 and 9'-10' cleavages of multiple carotenoid substrates to form a C14 dialdehyde and two C13 products, which vary depending on the carotenoid substrate (Schwartz et al. 2001). Some of the CCD1 apocarotenoid products have been reported to have antimicrobial activities (Fester et al. 1999; Goff and Klee 2006). Thus, it is possible that the expression of CCD1 in all tissues probably has a role in the formation of multiple antimicrobial compounds that may be important for plant defense. Above all, CCD1 has also been shown responsible for the synthesis of floral volatiles for attracting pollinating insects (Simkin et al. 2004).

3.3.5.3.1. Sequence analyses of CCDs from coriander leaves

CsCCD1 was 640 base pairs (bp) long and had an open reading frame (ORF) of 203 bp. Alignments of the deduced amino acid sequences showed that CsCCD1, shares high sequence identity with CCD1 of carrot as in Figure 3.16, since both belong to
Apiaceae family and homology from other plants particularly showed some similarity with Arabidopsis neoxanthin cleavage enzyme.

![Figure 3.16. Amino acid sequence alignment of CCD1 enzymes of carrot and coriander. The boxes are the mismatching bases in both coriander and carrot. Asterisks indicate completely identical amino acid residues. The protein sequences were aligned using the program ClustalW (http://embnet.vitalit.ch/software/ClustalW.html)](image)

Multiple sequence alignment of carotenoid dioxygenases (CCD1) amino acid sequence from carrot and coriander sequences showed 92% and 86% similarity with carrot and *Vitis vinifera* CCD1 sequences respectively. More than that, it showed 82% similarity with neoxanthin cleavage enzyme nc1 of *Arabidopsis thaliana*. Whereas it showed 75% similarity with tomato - *Lycopersicon esculentum* (synonym *Solanum lycopersicum*).
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Figure 3. 17. Phylogenetic tree of CCD1 genes constructed by the neighbor joining method using amino acid sequences by MEGA 6 software

Multiple sequence based phylogenetic tree, shown in Figure 3.17, reveals its genetic distance or closeness of relationship with other plant CCDs.

3.3.5.3.2. Changes in the expression levels of carotenoid dioxygenases (CCD1)

In plants, carotenoid degradation and apocarotenoid formation is determined by the action of mainly CCD1, CCD4 and NCED, whereas, from coriander leaves, a single CCD1 was identified. The expression of CCD1 was uniform in untreated (control) plants. In addition to that, it showed some amino acid sequence similarity with A. thaliana neoxanthin synthase. After treatment with MeJa, profound changes in the expression levels of CCD1 were observed, depending on the concentration of elicitor and expression periods (Figure 3.18). For 10 μmol/L MeJa, irregular expression patterns of CCD1 were observed, although a highest gene expression was observed on day 2. On 3rd day after treatment, decrease in expression occurred, with a further increase in the subsequent period. 100 μmol/L MeJa also showed similar pattern, except for a decrease on the second day. However, the higher concentrations of the elicitor had uniform expression in first three days and complete absence of expression after three days of treatment.
Figure 3.18. Gene expression analysis of *CCD1* (carotenoid dioxygenases) after treatment with methyl jasmonate. Highlighted structures are the possible substrates of *CCD1* and the apocarotenoid derivatives.
3.3.5.4. Quantitative Real Time polymerase chain reaction analysis of genes

Figure 3.19. Relative mRNA levels of 10 carotenoid biosynthetic pathway genes measured by quantitative real-time PCR. Relative expression level of Coriandrum

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*sativum*, carotenogenic genes after foliar application with plant growth regulator. A. *CsIPI*, isopentanyl pyrophosphate isomerase; B. *CsPSY*, phytoene synthase; C. *CsPDS*, phytoene desaturase; D. *CsZDS*, ζ-carotene desaturase; E. *CsCRTISO*, carotenoid isomerase; F. *CsLCYB*, lycopene β-cyclase; G. *CsLCYE*, lycopene ε-cyclase; H. *CsCHXB*, β-ring carotene hydroxylase; I. *CsCHXE*, ε-ring carotene hydroxylase; J. *CsCCD*, carotene cleavage dioxygenases. The height of each bar represents the mean of 5 measurements and error bars indicate the standard deviation. All transcripts values for individual genes were normalised with respect to the corresponding value of the 18s rDNA from *C. sativum* signal.

Relative mRNA levels of 10 carotenoid biosynthetic pathway genes measured by quantitative real-time PCR. **Figure 3.19** shows the differential expression of carotenoid pathway genes after foliar application with methyl jasmonate, analysed by quantitative RT PCR.

### 3.3.6. Carotenoid gene regulation

The regulation of carotenoid biosynthesis at the gene and enzyme level is poorly understood. A branched pathway such as that of carotenoid formation from isoprenoid precursors is not likely to be controlled by a single regulatory process. Instead, control points could exist at each branch point and probably be involved both at transcriptional and post transcriptional levels. Transcriptional regulation resulting in increased enzyme activities have also been illustrated in other plants such as tomato fruit ripening (Ronen et al. 1999).

Carotenoid accumulation may be potentially controlled by at least four different mechanisms. Firstly, the flux through the carotenoid pathway is controlled by rate-limiting enzymes. In various plants, different steps have been identified that control the biosynthetic pathway in this manner. In Arabidopsis, PSY enzyme was found to be responsible for the regulation of carotenoid biosynthesis (Rodríguez-Villalón et al. 2009). PSY was found to regulate the levels of 1-deoxy-d-xylulose 5-phosphate-synthase that produces precursors necessary for downstream metabolites of the pathway. Feedback regulation may therefore be an important process in carotenoid synthesis.
Secondly, the carotenoid pathway could be affected by other interacting pathways that divert/utilize certain substrates. For example, the MEP pathway produces GGPP, the substrate required for the first committed step of the carotenoid biosynthetic pathway. However, for the biosynthesis of other compounds such as chlorophylls, tocopherols, phylloquinones, and plastoquinones also utilizes GGPP as substrate, thereby for the allocation of the same substrate, a competition occurs. Light intensity appears to alter carotenoid formation by increasing the expression of certain carotenoid biosynthetic genes. Changes in \( LCY-\beta \) and \( LCY-\varepsilon \) mRNAs are also possible when changes in light intensity.

Thirdly, carotenoid accumulation can be affected by sink capacity because of the presence or absence of chloroplasts and/or chromoplasts. It has been shown recently, through the identification of a cauliflower Orange (Or) mutant, that increasing the metabolic sink capacity could increase carotenoid accumulation (Li et al. 2006). In most fruits, the ripening process is accompanied by the chloroplasts to chromoplasts conversion also coincides with the biosynthesis of carotenoids, making chromoplasts the major storage structures (sink) of these metabolites. This results in the sink capacity of these plastids as an important factor in the carotenoid accumulation. In Arabidopsis, carotenoid crystals form as an alternate sink for accumulating carotenoids (Maass et al. 2009) there is no plastid conversion to chromoplasts. Finally, carotenoid degradation has been shown to impact final carotenoid concentrations; in particular the activity of a group of enzymes, the carotenoid cleavage dioxygenases (CCDs). Down-regulating \( CCD \) activity affects carotenoid levels in roots of Medicago (Floss et al. 2008) and potato tubers and flowers (Campbell et al. 2010). Studies in Arabidopsis seeds, strawberry (\( Fragaria \) ananassa), grape (\( Vitis \) vinifera L.) and citrus fruits as well as chrysanthemum (\( Chrysanthemum \) morifolium) petals, have all demonstrated that the carotenoid pool is determined in part by the rate of degradation by carotenoid cleavage dioxygenases, which seem to have different substrate preferences.

Herbicidal inhibitors are compounds that inhibit processes essential for plant growth. For investigating plant gene functions these inhibitors can serve as excellent tools because enzymes targeted by herbicidal inhibitors are essential for plant growth
and development. Consequently herbicidal inhibitors have been helpful not only in the identification of target site genes, but also in facilitating understanding of the regulatory controls of plant metabolic pathways in general. Elucidation of the mechanisms that regulate this biosynthetic route is critical as is the further biochemical characterization of particular enzymes that participate in this pathway. Due to the central role this pathway plays in the growth and development of plants as well as for reaping higher concentrations of these metabolites for human health and nutrition, the identification of the major regulatory steps is essential. An in depth knowledge of the regulatory mechanisms of the pathway will also benefit the biotechnological production of commercially important isoprenoids, such as carotenoids. Accordingly, the commonly used carotenoid pathway inhibitors were used in the present study, which are known to regulate three important checkpoints in the pathway. Both at the gene expression and product levels were determined after each inhibitor treatment by using qRT-PCR and HPLC methods.

3.3.6.1. Fosmidomycin an inhibitor of MEP pathway (Non mevalonate pathway)

The upstream pathway enzymes in the MEP pathway have also been shown to influence carotenoid biosynthesis (Enfissi et al. 2005) and there is an enhanced flux through the pathway in Arabidopsis plants overexpressing the **DXR** gene (Carretero-Paulet et al. 2006). Fosmidomycin and a range of environmental factors were applied to modulate MEP pathway. In the present study, coriander shoots when exposed to 200 µmol/L fosmidomycin caused inhibition of carotenoids accumulation, which was apparent on third day onwards. Once an appropriate concentration of each inhibitor was selected, further experimentation was continued to assess its effects on the synthesis of carotenoids, β-carotene, lutein and RNA in coriander leaves grown under controlled conditions. Lower concentrations, especially concentrations below 150 µmol/L, fosmidomycin had no influence on the biosynthesis of β-carotene. **Figure 3.20** presents the HPLC profiling of carotenoids in coriander leaves treated with fosmidomycin (an MEP pathway) inhibitor after 2 days and 5 days of treatment with respect to the control **Figure 3.20. A.**
Figure 3.20. HPLC profiling of carotenoids in coriander leaves treated with fosmidomycin (an MEP pathway) inhibitor. A. Control. B. After 2 days. C. After 5 days of treatment.
After foliar spray with fosmidomycin, expressions of major carotenogenic genes were quantified. This inhibitor also exhibited similar pattern of reduction in β-carotene and total carotenoid content. Lutein concentration also reduced as the time progressed as in the Figure 3.21. The effect of this inhibitor was much faster than the other inhibitors used. Major genes influenced by the inhibitor were PDS, ZDS and CRTISO. LCYE also showed decreased expression. Whereas, CHXB showed no variation in the expression pattern, which is known to participate in the formation of β-carotene derived xanthophylls such as violaxanthin and neoxanthin. IPI is one of the upstream enzymes in the MEP pathway, but exhibited no variation in its expression up on inhibitor treatment, when compared with control plants.

3.3.6.2. Norflurazon – a phytoene desaturase (PDS) inhibitor

The contribution of carotenoid biosynthesis to total carotenoid levels was studied by treating coriander plants at young stage (15 to 25 days) with 100 μmol/L norflurazon (NF); a herbicide that blocks carotenoid biosynthesis by inhibiting PDS leading to an accumulation of phytoene. HPLC analysis of carotenoids after inhibitor treatment showed the decreased content of total carotenoids and both lutein and β-carotene compared with untreated control (Figure 3.22).
Figure 3.22. Changes in carotenoid profile of coriander leaves after treatment with herbicide norflurazon (a phytoene desaturase inhibitor). A. Control; B. 1 day after treatment; C. After 3 days; D. After 10 days

Treatment with this inhibitor did not exert an immediate bleaching effect; however, after three days leaves started losing chlorophyll from the periphery towards the centre. As the time progressed, both carotenoids and chlorophyll levels decreased, as shown in Figure 3.22. B, C, and D compared with untreated control Figure 3.22. A. β-carotene concentration decreased much faster than lutein and other xanthophylls. Unexpectedly, other xanthophylls such as violaxanthin and antheraxanthin enhanced. As the treatment time progressed there was a substantial decrease in the expression of PDS gene. Other important genes that showed decrease in their expressions were ZDS and LCYB gene. But the expression of CRTISO decreased after 7th day Figure 3.23. A. Norflurazon inhibited both PDS and ZDS. Probably both these genes are controlled by same regulatory factors. In coriander, enhancement of carotenoid biosynthesis occurred through the enhanced expression of genes coding for PDS and ZDS, inferring that a decrease in expression of PDS and ZDS along with other genes controls the flux through this pathway in coriander, which is an important observation made in the present study.
Figure 3.23. Effect of phytoene desaturase inhibitor on the carotenoid biosynthetic pathway enzymes of coriander. 
A. Expression profiles of major genes; 
B. Carotenoid pathway showing norflurazon targeting PDS; 
C. Carotenoid profiling after amitrol treatment; 
D. Treated leaves after 15 days
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LCYE is the major determinant of α branch of the pathway. The LCYE expression reduced to very low level towards longer time periods and initial time periods it remained almost uniform. Substrate of phytoene desaturase (PDS), phytoene was not detected in the HPLC chromatogram. Lycopene is the substrate for both lutein and β-carotene- major carotenoids present in coriander leaves and the levels of these carotenoids were determined by the activities of β and ε-cyclases. After norflurazon treatment slight changes in the expression of LCYE observed. But the lutein content was decreasing, indicating that upstream genes control the carotenoids pathway in coriander at different levels.

3.3.6.3. Amitrol-cyclization inhibitor of lycopene

In coriander leaves, lycopene cyclase inhibitor, amitrol changed the carotenoid profile in a different manner. After three days of treatment, leaves showed pale yellow color. Total carotenoids and lutein remained constant whereas β-carotene decreased on the day one, following a decreasing trend afterwards. Lycopene is the substrate for both the cyclases, although in HPLC analysis (Figure 3.24) lycopene could not be detected.

![Figure 3.24. HPLC profiling of major carotenoids (lutein and β-carotene) concentrations after treatment with amitrol after 7 days (B) as compared with control (A)](image)

Amitrol is the lycopene cyclase inhibitor, so the gene expression pattern of both LCYB and LCYE were determined thought out the treatment time. Both LCYB and LCYE decreased after amitrol treatment. CHXB transcript level was uniform as in other
Figure 3.25. Effect of lycopene cyclase inhibitor on carotenogenesis. A. Effect of amitrol on carotenoid content; B. Pathway genes $LCYB$ and $LCYE$ targeted by amitrol; C. Gene expression profiling; D. Amitrol treated (after 5 days)
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treatment responsible for the reduced concentration of β-carotene. After three days of treatment, a significant reduction in the expression of PDS gene was observed. A corresponding decrease in total carotenoids with high lutein may be the result of feedback inhibition by the product. Towards the end of the treatment time, β-carotene peak was eluted with another peak, it may be a degradation product of β-carotene or may be a pathway intermediate with the same absorption maxima. Significant reduction in the chlorophyll a and b was also observed as shown in the Figure 3.24. After three days a substantial reduction in the expression of PDS gene was observed (Figure 3.25.B) and with a corresponding decrease in total carotenoid with high lutein, may be the feedback inhibition by the product. Carotenoids and chlorophylls were quantified and their compositions were analyzed with biochemical and the gene expression analysis revealed its correlation with the latter. The treatment with amitrol, which interrupts lycopene cyclization, still permits the plants to produce xanthophylls and some amount of chlorophylls, indicating that the inhibition is probably incomplete. In order to protect the photosynthetic machinery, other xanthophylls such as antheraxanthin were also enhanced.

3.4. Discussion

Carotenoid related research is mostly focused on model organisms such as Arabidopsis, maize and tomato, with a very limited number of studies conducted in vegetables. Therefore, such studies will have a huge impact on knowledge about carotenoid regulation, offering an opportunity for their genetic improvement (Giuliano et al. 2008). Even though coriander is a routinely used GLV and a good source of carotenoids, carotenoid biosynthetic pathway gene expressions have never been studied. The present study is the first report that evaluated the expression levels of ten carotenoid pathway genes in coriander leaves under normal and elicited conditions. Jasmonic acids and MeJa naturally affect a wide range of cellular activities, primarily because they perturb signaling pathways. Jasmonates play a critical role in plant defense mechanisms against pathogens and insects, and modulate secondary metabolite biosynthesis, through regulation of defense-related genes expression (Browse 2009).
3.4.1. Differential expressions of carotenoid genes upon methyl jasmonate treatment

Carotenoid accumulation in coriander leaves is both time and concentration dependent. Higher concentration of MeJa has an immediate effect on major carotenogenic genes expression whereas, 10 µmol/L concentration of this elicitor enhanced the expression of ten carotenogenic genes with highest transcript level of genes CsIPI (10fold), CsPSY (3.7fold), CsPDS (11fold), CsZDS (9fold), and CsCHXE (5fold) after 48 h of control plants. Maximum level of transcripts observed on the 3rd day after foliar application. Different concentrations of MeJa have different levels of impact on the expression of carotenogenic genes, of which there is no significant difference in the expression of CsCHXB and CsLCYB. In response to all the three concentrations of MeJa used, there was only a slight increase in expressions of genes - CsCHYB and CsLCYB, which are genes responsible for the transcriptional regulation of β-carotene, suggesting that its production is tightly controlled by the expression of upstream genes of carotenoid biosynthetic pathway genes. Since high concentrations of MeJa showed an inhibitory effect on carotenoid accumulation and lower concentration (10 µmol/L) could be used for the carotenoid enhancement. Among the treatments, for 100 µmol/L MeJa there was no significant difference in the expression levels of CsPSY, CsBCHY, CsLCYB, but five, three, and two fold increase in the expression of CsIPI, CsZDS, CsPDS genes respectively.

Carotenoid accumulation in coriander leaves occurred chiefly by the stress induced up regulation of carotenogenic genes. Although, all ten carotenogenic genes up regulated upon MeJa treatment, differential expression of each gene occurred in response to each concentration of this compound. Another study in tomato revealed that, during fruit maturation, PSY and PDS have important roles in controlling the flux in the carotenoid biosynthetic pathway; increased expression of these genes is correlated with accumulation of lycopene (Cunningham 2002) and total carotenoid content. In Arabidopsis, disruption of PDS decreased carotenoid biosynthesis (Qin et al. 2007). Phytoene synthase is the rate-limiting enzyme in carotenogenic pathways in many plants, whereas in coriander shoots, phytoene desaturase and ζ-carotene desaturase enzymes appear to be the rate limiting enzymes, since their expressions were
significantly altered. Transgenic tomatoes expressing \textit{PDS} gene were found producing 2–4 fold \(\beta\)-carotene and other carotenoids, lutein, neoxanthin, zeaxanthin (Römer et al. 2000). Same results were obtained with tobacco and rice, where PDS expression correlated with the enhancement of \(\beta\)-carotene accumulation in these plants (Misawa et al. 1994). In the present study with coriander, MeJa (10 \(\mu\)mol/L) appears to significantly influence the gene transcriptions, since it was observed that the treatment enhanced the level expression of genes such as \textit{CsPDS}, \textit{CsZDS}, by 11 fold, 9 fold respectively, as compared to control. This change in the expression levels is also indicative of the fact that upstream pathway genes such as \textit{PDS} and \textit{ZDS} may play pivotal role in the regulation of carotenoid accumulation in coriander. Through a previous study (Divya et al. 2014), it was demonstrated that the carotenoid content in coriander leaves could be substantially enhanced by treating with plant hormones MeJa and SA. The set of experiments in this chapter further substantiates such observations through molecular regulation data of ten carotenogenic pathway genes.

The gene expression of \textit{LCY-}\(\beta\) (\textit{LCYB}) and \textit{LCY-}\(\epsilon\) (\textit{LCYE}) controls the flux through the \(\beta\)-carotene and \(\alpha\)-carotene pathway branches. Lutein content reduced dramatically during apple fruit development when \textit{CsLCY-}\(\epsilon\) was down regulated indicating that \textit{CsLCY-}\(\epsilon\) expression is also crucial in contributing to the total carotenoid content (Ampomah-Dwamena et al. 2012). In this study, lutein content remained unchanged, which directly correlated with the expression of \textit{LYCE} and reduction in lutein content was also consistent with the down regulation of \textit{LYCE} gene.

\textit{CsCCD1} is a cytosolic enzyme and since it lacks a signal peptide for the transport to the plastid, whereas; carotenoids are synthesized in the plastids. Therefore, the cytosolic \textit{CsCCD1} enzymes have access only to those carotenoids distributed on the outer envelope of plastids or has access to only partially degraded carotenoid released from plastid, which might be released after the destruction of plastid. In some plants, \textit{CCD1} expression levels were correlated to ripening with a concomitant decrease in lutein content in strawberry (García-Limones et al. 2008). In maize highest expression of \textit{CsCCD1} transcripts and the reverse relationship of \textit{CsCCD1} expression and carotenoid levels was observed (Vallabhaneni et al. 2010). Simkin et al. (2004a) reported significant expression of \textit{LeCCD1} in tomato leaves and roots. A similar
observation was attributed to the expression of \textit{AtCCD1}, where oxygen-containing carotenoids- xanthophylls appeared to serve as better substrates for \textit{AtCCD1} than β-carotene itself (Schwartz et al. 2001). In previous studies in Arabidopsis, altered \textit{AtCCD1} levels did not have an impact on leaf carotenoid accumulation although the \textit{AtCCD1} gene was actively expressed in leaves, whereas in the seeds, correlations between the increases in \textit{AtCCD1} expression with concomitant decrease in carotenoid substrates have observed (Auldridge et al. 2006). In another study in \textit{Crocus sativum}, Rubio et al. (2008) reported the expression of \textit{CsCCD1a} as a response to different stress treatments, and that \textit{CsCCD1} transcript levels were up regulated by senescence. MeJa up regulated the expression of stress and secondary metabolism associated proteins, and 6-fold enhancement of ζ-carotene desaturase were observed in Arabidopsis leaves (Chen et al. 2011), which correlates with the current observation in coriander. While working with peach cultivars, Pirona et al. (2012) reported that the expressions of carotenoid cleavage dioxygenases, phytoene synthase, ζ-carotene desaturase were differentially regulated and that their expressions correlate with genes involved in the formation of volatile compounds.

In MeJa (10 µmol/L) treatment, the mRNA expression of \textit{CsLCYB} was maximum at day 1, which preceded the fast accumulation of β-carotene and lutein, indicating that this gene might up regulate the accumulation of carotenoids at post-transcriptional level. Whereas, a maximum transcript level of other genes – \textit{CsPSY} and \textit{CsPDS} that occurred in 3\textsuperscript{rd} day was in accordance with carotenoids accumulation, suggesting the transcriptional level control of these genes. The expression of \textit{CsLCYE}, \textit{CsBCHY} and \textit{CsIPI} was prior to the quantitative accumulation of β-carotene, suggesting their participation at transcriptional level.

3.4.2. Expression of main carotenoid pathway genes and pigment production under elicited conditions

Chloroplast-type carotenoids are represented by lutein, violaxanthin, and β-carotene that are essential for photosynthesis, and lutein, the most abundant carotenoid in coriander leaves. Lycopene was not detected in the HPLC analysis, probably due to
the complete utilization of this substrate for the synthesis of downstream carotenoid products.

Gene expression levels determined by the qPCR methods were found to correlate with the concentration of total carotenoids, β-carotene and lutein. While both β-carotene and lutein were highest on 3rd day, only β-carotene showed a decreasing trend afterwards. Genes responsible for the lutein formation in the ε-branch of the pathway such as CsLCYE and CsCHXE were differentially expressed after treatment with MeJa at different concentrations. At 10 μmol/L concentration of MeJa, the expression of CsLCYE and CsCHXE were similar and correlated with lutein content. But at 500μmol/L of MeJa, CsCHXE expression varied with treatment time, showing a complete absence of expression after three days of treatment. The expression of CCD1 gene, one of the key genes responsible for the carotenoid breakdown mechanism, also decreased after 3rd day of foliar treatment with MeJa. This might be because of the feedback inhibition of CCD1 gene by lutein or other apo-carotenoid products in coriander leaves. Further work needs to be done to elucidate the mechanism of end-product regulation. Since lutein is an important substrate for CCD1 gene, its complete absence of expression in response to 500 μmol/L of MeJa may be responsible for the consistent level of lutein after 3rd day. For 500 μmol/L MeJa, only CsLCYE expressed fourfold after 3rd day, and all other genes were expressed only in the initial stages of treatment. Thus, the enhanced expression of CsLCYE, and the complete absence of CCD1 expression may be responsible for the higher production of lutein in the absence of CsCHXE. The decrease in the concentration of β-carotene observed towards later stages of treatment with 10 μmol/L and 500 μmol/L of MeJa suggests that β-carotene may be the possible target of CCD1 because its expression was high throughout for all treatments and periods. Although there are a few reports on the senescence promoting effects of MeJa, the author’s previous study demonstrated that in case of mature leaf stage (before flowering) in coriander, only 1000 μmol/L MeJa promoted senescence.

This study can be considered as a first step in understanding the mechanisms of regulation of carotenoids synthesis in coriander leaves. Identifying the differential expression proteins under MeJa induction and determining the relationship between carotenoid biosynthesis genes and the proteins will give more insight. Further work will
be needed to examine the possibility of allelic differences of major genes, different regulatory mechanisms such as feedback inhibition, epigenetic regulation, rate of degradation by carotenoid cleavage dioxygenases or posttranscriptional regulation and their relationships with carotenoid accumulation. The current study also provides a platform for the in-depth research on the regulation of carotenoid genes, which could be useful for the genetic improvement program especially in leafy vegetables and probably in other important food crops.

3.5. Effect of inhibitors on carotenoid content and gene expression

3.5.1. MEP pathway inhibitor fosmidomycin

The studies concerning the early steps of carotenogenesis are rare and there is less information regarding whether the isoprenoid substrate can be influenced by carotenogenic conditions or selective inhibitors fosmidomycin. This approach can be useful to produce specific carotenoid by precursor regulation such that it allows modulated flow of precursors downstream for other isoprenoids and carotenoids. Therefore, by using data of the present study, by use of inhibitors, pathway regulation for biosynthesis of β-carotene and other carotenogenesis is made possible, without the intervention of genetic engineering method. By evaluating the isoprenoid-β-carotene intervention approach, the action of inhibitors it has been demonstrated the possibility of programming the early steps of carotenogenesis leading to the accumulation of β-carotene in coriander leaves. 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway for the biosynthesis of plastid isoprenoids (including carotenoids) has not been fully elucidated yet, despite it being a central metabolic pathway for plant life. Fosmidomycin (200 µmol/L), an inhibitor of 1-deoxy-D-xylulose-5-phosphate reducto isomerase (DXR), suppresses the biosynthesis of isoprenoids and accumulation of carotenoids (β-carotene) respectively in the non-mevalonate (MEP pathway). Fosmidomycin influenced the expression of overall biosynthetic pathway starting from IPI to the ε and β hydroxylases. Expression pattern was almost similar to the desaturation inhibitor norflurazon, in which both upstream and downstream pathway genes were down regulated. This may be because of the reduced availability of precursor molecules. The highly affected ones were those of the upstream genes *PDS,*
ZDS and CRTISO. Downstream genes such as LCYE were also down regulated. The reduction in total carotenoid, β-carotene and lutein could be the reduced expression of PDS resulting in the reduced flux of precursor flow through the entire pathway. Earlier studies demonstrated the role of DXR and plastid isoprenoids in tomato fruit ripening by blocking DXR activity (Rodríguez-Concepción and Boronat 2002; Schwender et al. 1999; Sharkey and Yeh 2001). Fosmidomycin, a strong and specific inhibitor of plant DXR, inhibited DXR activity and that plastid isoprenoid biosynthesis was found essential for tomato fruit carotenogenesis. Rodríguez-Concepción et al. (2001) reported that treatment with fosmidomycin, however, significantly induced the accumulation of certain transcripts from genes involved in carotenoid biosynthesis such as DXR, DXS and PSY1 which is correlated with our investigation that initial genes IPI and PSY transcript levels were uniform throughout the treatment time.

3.5.2. Norflurazon (NF) mediated inhibition of PDS gene

NF inhibited phytoene desaturase (PDS). It was also observed in this study that after 48 hour of treatment, there was a decrease in the content of both lutein and β-carotene in treated plants and that the inhibitor did not cause an immediate bleaching effect. NF treatment in the light did not lead to phytoene accumulation (substrate of phytoene desaturase) as observed though data of HPLC analysis. After elicitor treatment PDS gene transcript level was the highest, and there was a concomitant decrease in the expression of PDS after NF treatment. Where the expression levels of other genes were also decreased, especially those responsible for lutein and β-carotene (LCYE and LCYB). This, as stated above, can be explained by a reduced carotenoid flux in to the pathway. Simkin et al. (2003) reported a decrease in expression level of PDS and ZDS upon norflurazone treatment in dark conditions. Species to species variation has been observed in some plants such as Arabidopsis (Wetzel and Rodermel 1998) and pepper (Simkin et al. 2000). The reason for these variations is not clear. Since the regulation of carotenogenesis is apparently controlled by several regulatory mechanisms, it may be hypothesized that some of the mechanisms are predominant over others, which differ from plant to plant as well as different organs in the same plant.
3.5.3. Cyclization inhibitor amitrol

As in other GLVs, lutein is the predominant carotenoid in coriander leaves, which was followed by β-carotene. Amitrol treatment caused substantial changes in the pathway of coriander than other inhibitors since it is a cyclization inhibitor, targeting mainly the cyclization enzymes \textit{LCYE} and \textit{LCYB}, and hence their activity could limit the carotenoid and xanthophyll content in plants. This particular inhibitor, amitrol showed a huge effect on both plant growth and pigment content. The inhibitor induced identical visible symptoms in treated plants; characteristically, subsequent to herbicide application a bleached, chlorotic appearance of tissue was observed. Even though there was a major change in the carotenoid pattern with much change in the β-carotene, the total carotenoids and lutein remained unaffected. After two to three days of treatment, apart from the cyclization enzymes, it affected the upstream genes such as PDS gene also, which is the key regulatory gene of the carotenoid pathway. After 7 days of treatment, lutein content decreased significantly, whereas β-carotene reduced immediately after treatment and concomitant increase in other β-carotene derived xanthophylls such as antheraxanthin and violaxanthin. This may be because of the highest expression level of \textit{CHXB} throughout the treatment time. Lutein content decreased only after the reduction in the expression of upstream genes of the pathway. HPLC analysis revealed no accumulation of lycopene or other intermediates such as phytoene and phytofluene, which is also reported in other plants. In the present study, treatment with amitrol leads to the degradation of β-carotene and towards the end stages of treatment. Absence of lycopene may be the possibility of an alternate pathway for the synthesis of β-carotene and xanthophylls, which bypasses lycopene cyclization in leaf chloroplasts as reported in tomato chromoplasts and Arabidopsis chloroplasts (Ronen et al. 2000; Pecker et al. 1996; Cunningham et al. 1996). The activity may be dependent upon high temperature.

3.6. Conclusion

The foliar application of MeJa and SA enhanced biologically active compounds such as total carotenoids, β-carotene, lutein, chlorophylls and phenolics. SA treatment, at high concentration (500 μmol/L) elicited more of β-carotene and lutein, whereas
MeJa was efficient at a very low concentration (10 μmol/L). Also, higher than 10 μmol/L of MeJa suppressed carotenoids accumulation, promoting senescence at later stages. Highest elicitation of total phenolics (fourfold) was observed after 24 h with 10 μmol/L of SA treatment, which resulted in sevenfold increase in chlorogenic acid as well. Overall, each elicitor treatment was effective at a particular growth stage, viz., rapid growth stage occurring before the initiation of flowering, whereas at later stages (7th and 12th day) no significant elicitation was observed. More research is needed to elucidate the mechanisms involved in elicitor-mediated enhancement of carotenoids and phenolic compounds.

Three concentrations of MeJa treatments stimulated main carotenoids accumulation in coriander, in which 10 µmol/L MeJa induction have an optimal effect in our experiment. The enhanced level of carotenoids accumulation was probably due to the up-regulation of nine carotenoid genes. In this study, it was found that the carotenoid content and the differential expression of carotenogenic genes after treatment with MeJa. The transcript amount reached its maximal value on 3rd day for most of the genes. \(CsPDS, CsZDS, CsLCYE, CsIPP\) and \(CsCHX\) are the key genes, which regulates the flux through the pathway. The expression carotenogenic genes correlated with the β-carotene and lutein content after treatment with MeJa. The enhancement of lutein and β-carotene was due to the increase in the expression and complex regulation of four carotenoid pathway genes. Results indicated the occurrence of an extensive network of coordination and regulatory interactions induced by plant hormone treatment leading to the enhancement of phytonutrients. The present study reports the changes in gene expression, signaling, stress responses, pigment biosynthesis, closely links the growth regulator mediated stress response mechanisms in plants. The information generated in this study can be used as a starting point for the subsequent research for establishing the connection between differentially expressed genes and the protein products involved in the carotenoid enhancement in GLVs.

To elucidate the regulation of carotenoid pathway genes, inhibitors targeting the regulatory nodes in the pathway were studied, confirming that, the precursors for the carotenoids pathway are the products of MEP pathway rather than the non mevalonate pathway. In many plants, \(PSY\) is the key regulator of overall carotenoids content,
whereas in coriander *PDS* was the one which regulate the overall flux through the pathway along with *ZDS* as evidenced from the norflurazon treatment. Third key regulatory node targeted was the cyclization step of lycopene, which will control the β and ε branch of the pathway, which inhibited *LCYB, LCYE* and at later stages regulates the upstream genes also. The content of β-carotene decreased immediately after amitrol treatment and such decrease was more than that of lutein, although the total carotenoids remained unchanged. However, towards the last stages of treatment period, lutein decreased with concomitant increase in lutein epoxides and β-carotene degradation products. All these clearly establish that, the regulation of carotenoids is possible to achieve by external treatments, rather than genetic modification methods.

**Chapter highlights**

- Various abiotic elicitors were screened and the best elicitor was selected for the enhancement of carotenoids.
- Differential expression of key carotenoid biosynthetic genes were established after elicitor treatment.
- Key regulatory gene, for the enhancement of xanthophyll and β-carotene was determined.