Transcriptional Regulation of Chlorogenic Acid Biosynthesis in Carrot Root Slices Exposed to UV-B light.

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Abstract

Orange carrots are well known for their nutritional value as producers of β -carotene, a vitamin A precursor. Lesser known, is their ability to accumulate antioxidants such as chlorogenic acid. Chlorogenic acid is produced through the same biosynthetic pathway that produces lignins, anthocyanins, flavonols and isoflavonoids, the phenylpropanoid pathway. This pathway can be induced by UV-B light and wounding to produce large amounts of chlorogenic acid. We identified a number of the carrot structural genes in this pathway and their proposed regulators in carrot root slices and analyzed their expression in response to UV-B light exposure using real-time PCR. Our results indicate that not only does the expression of the structural genes correlate with their proposed regulators, including *DcHY5* and others, but also correlate with chlorogenic acid production. Our results indicate that the HY5 - UVR8 signaling network may exist and function in carrot roots.

Introduction

A positive correlation between diets containing antioxidant rich foods and a reduced risk of some cancers and cardiovascular disease exists (Joven et al., 2013). The tissue specific accumulation of reactive oxygen species within the human body is one potential pathway to these diseases and it is theorized that antioxidants defend against these diseases by reducing the levels of these reactive

oxygen species (Sawa et al., 1999; Xu et al., 2012; Joven et al., 2013; Oboh et al., 2013).

Nutritionists and plant biochemists recently focused their attention on chlorogenic acid (CGA) because of its antioxidant properties and presence in multiple crops such as eggplants, tomatoes, coffee, artichokes, carrots, apples, pears and plums (Fleuriet and Macheix, 2003; Niggeweg et al., 2004; El-Seedi et al., 2012). CGA is bio-available either as itself or after cleavage during digestion, as caffeic acid, an equivalent antioxidant (Olthof et al., 2001; Sato et al., 2011). In addition to its antioxidant properties, Thom et al., (2007) reported that consumption of a CGA enriched instant coffee reduced glucose absorption and body mass of 30 overweight subjects.

Arabidopsis thaliana mutants with lowered levels of flavonoids and sinapic acid esters show enhanced stress to UV-B irradiation indicating the importance of these UV-B absorbing compounds in protection of plants (Li et al., 1993). In carrot (*Daucus carota*) roots, CGA accumulation is influenced by stresses such as UV-B irradiation and wounding (Avena-Bustillos et al., 2012; Du et al., 2012; Jacobo-Velázquez and Cisneros-Zevallos, 2012; Surjadinata and Cisneros-Zevallos, 2012). Additionally, chlorogenic acid accumulation in tomato improved UV-tolerance (Clé et al., 2008). Plants sense UV-B light through the UV-B receptor, UVR8 and possibly a tetrahydrobiopterin containing receptor and initiate a response through HY5, a bZIP transcription factor that induces the response genes (Heijde and Ulm, 2012; Takeda et al., 2014). In *Arabidopsis* seedlings, two independent responses to UV-B illumination are possible depending on the length of exposure, 24 or 96 hrs, with an early response enhancing anthocyanins and tocopherol and a later response enhancing flavonols and expression of senescence-associated genes (Kusano et al., 2011). Wounding alone can cause an increase in phenylalanine ammonia-lyase (PAL) activity with concomitant accumulation of CGA and dicaffeoylquinic acids in carrots (Jacobo-Velázquez and Cisneros-Zevallos, 2012). Wounded carrots increase PAL activity upon UV-B illumination, however

continuous white light illumination causes hairy root cultures of carrot, normally grown in dark, to turn green and reduce PAL activity in an apparent redirection of metabolite precursors from phenylpropanoid biosynthesis toward isoprenoid biosynthesis (Avena-Bustillos et al., 2012; Mukherjee et al., 2016). CGA is produced from phenylalanine through a series of reactions by the enzymes, PAL, trans-cinnamate 4-monooxygenase (C4H), 4-coumarate: CoA ligase (4CL), hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase (HCT), hydroxycinnamoyl CoA quinate transferase (HQT) and 4-coumarate 3-hydroxylase (C3H) (Figure 1). Investigations of CGA production in plants have relied predominantly on measurements of phenylalanine ammonia-lyase (PAL) activity because this is a key step in phenylpropanoid biosynthesis (Klaiber et al., 2005). The early part of this pathway is common to many classes of phenylpropanoids. 4-Coumaric acid is converted to 4-hydroxybenzoic acid (4-HBA) and accumulates in the cell wall in large amounts in hairy root culture of carrot (Sircar et al., 2007; Sircar et al., 2009; Figure 1). 4-Coumaroyl-CoA resulting from 4CL action on 4-coumarate is a key intermediate for many classes of these compounds including, stilbenes, isoflavonoids, anthocyanins, suberin, rosmarinic acid, lignins and CGA. C4H and 4CL were induced in parsley cells exposed to light (including UV), fungal elicitor and wounding (Logemann et al., 1995). Shredded carrots were recently used to indicate wounding induction of PAL, 4CL and other genes involved in anthocyanin production (Jacobo-Velázquez et al., 2015; Becerra-Moreno et al., 2015). Other enzymes involved in CGA biosynthesis including HCT, HQT and C3H have been studied in artichoke and coffee (Lepelley et al., 2007; Mahesh et al., 2007; Sonnante et al., 2010; Clé et al., 2008). Chief transcriptional regulators of the phenylpropanoid pathway in many plant species appear to be members of the MYB and basic helix-loop-helix (bHLH) families of transcription factors (Kusano et al., 2011; Luo et al., 2008; Maeda et al., 2005). Carrot DcMYB1 has been shown

to be an important transcriptional activator for *DcPAL1* and DcMYBs 3 and 5 are known to interact with the *DcPAL3* promoter (Maeda et al., 2005; Wako et al., 2010). In *Arabidopsis*, an important bZIP transcription factor induced by UV-B irradiation via UVR8, AtHY5, is a positive regulator of light and UV-B responses and may be a signal transducer for other processes (Ulm et al., 2004; Brown et al., 2005; Zhang et al., 2011). In fact, *Arabidopsis* HY5 mutants have a number of defects including altered root morphology (Oyama et al., 1997). Because CGA may be important for human and plant health, manipulation of CGA content in crop plants is important to further improve nutritional value of these plants. Our research seeks to reveal regulation of CGA content through gene expression so that we may determine the correct avenue for that improvement. Here we identify genes involved in CGA biosynthesis and examine their expression and some of their possible transcriptional regulators such as MYB TFs and putative AtHY5 and AtUVR8 homologs in carrot.

Materials and Methods

Plant Material and UV-B Treatment

Whole carrots (*Daucus carota*) were harvested by Grimmway Farms in Bakersfield, CA, placed in insulated cardboard boxes with carrots wrapped in plastic bags and ice-packs over them, and sent by overnight shipping to Western Regional Research Center at Albany, CA. Carrot roots were washed and sliced into 3 mm thick discs with a Hobart model 1612P (Hobart Corp., Troy, OH) slicer using the middle section of the root resulting in disc diameters of 2.2 to 2.6 cm. Discs from 3 different carrots were prepared and kept on two separate Petri dish (100 x15 mm, Fisher Scientific) plates for each carrot, treatment and control, throughout the procedure. After slicing, one disc from each control plate was immediately frozen in liquid nitrogen and stored at -80 ° C for the 0 h T₀ timepoint.

A UV conveyor 40 dual lamp curing system equipped with two SunRay 400 SM UVB flood lamps (Uvitron International Inc., West Springfield, MA) was used at one constant conveyor speed and lamp height to achieve one constant UV-B dose treatment. UV-B dose (energy) and intensity (power) was measured using an Uvicure Plus II radiometer (EIT Inc., Sterling, VA). Exposure time and speed were also measured and recorded. The top surfaces were UV-B treated to a total energy dose of 120 mJ/cm^2 in 14 s with a bimodal peak irradiance UV-B light of 18.7 mW/cm^2 . The samples were flipped over and the treatment repeated. Samples were taken immediately after treatment from the treatment plates for the 0 h T_1 . Sample plates were then placed at 15°C and 45% RH in the dark for six days. A set of plates containing untreated disc samples was also placed at these conditions. Samples were taken from both sets of plates at 2 h (T_2), 1 day (T_3), 2 days (T_4), 3 days (T_5), and 6 days (T_6) at approximately the same time each day.

HPLC Materials, Chemicals and Analysis

Acetonitrile (HPLC grade), methanol (HPLC grade), citric acid, and ammonium acetate (enzyme grade) were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals used in the chemical analysis were obtained from Sigma-Aldrich (St. Louis, MO). Water was deionized to $\geq 18.1 \text{ M}\Omega/\text{cm}$ resistance using a Barnstead NANOpure Deionization System (Dubuque, IA). HPLC mobile phases were filtered through a $0.45 \mu\text{m}$ type nylon membrane filter (Millipore, Billerica, MA) prior to use. The HPLC system consisted of a Waters 2695 Separations Module (Waters, Milford, MA) coupled to a Waters 996 PDA detector and ESA CoulArray Electrochemical detector (Chelmsford, MA). We used the chromatographic and electrochemical detection settings of Du et al., (2012). In short, the chromatography was accomplished using a gradient consisting of (A) 25 mM citric acid, 25 mM ammonium acetate in 90:10 H_2O :acetonitrile and (B) 25 mM citric acid, 25 mM ammonium acetate in 50:50 H_2O :acetonitrile. Analyses were conducted at 30°C using a Kinetex

150 x 4.6mm (Phenomenex, Torrance, CA) column equipped with a Phenomenex AFO-8497 guard column. The injection volume was 20 μ L and the flow rate was 1mL per minute. Extracts were filtered thru a 0.22 μ m Millipore Millex-GV syringe filter before injection. Stock solutions of the chlorogenic, caffeic, coumaric, and ferulic acids were prepared in 50:50 methanol:water and diluted with water to obtain working solutions covering the concentration range of 1.0 μ g /mL to 150 μ g/mL for chlorogenic acid and 20 ng/mL to 1000 ng/mL for caffeic, coumaric, and ferulic acids, respectively. Concentrations were determined using external calibration curves and reported as ng/g fresh weight.

Sequence Analysis

Sequence analysis and visualization was performed using CLC Free Workbench (currently “CLC Sequence Viewer” at <http://www.clcbio.com>). Primers were designed based on the EST contig or GenBank sequence using AMPLIFY 3 software (<http://engels.genetics.wisc.edu/amplify/>). When possible, primers were designed across intron/exon junctions to prevent amplification of genomic DNA sequences.

Real-time RT-PCR

Total RNA from carrot root tissue (discs) was extracted using TRIzol®plus PureLink™ RNA Mini Kit, catalog number 12183-018A (Invitrogen, Life Technologies, Carlsbad, CA), quantified spectrophotometrically and cDNA was synthesized using GeneAmp®RNA PCR kit, catalog number N8080017 (Applied Biosystems, Foster City, CA) in accordance with manufacturer’s protocol. 1 μ L of diluted cDNA (1in10) was used in each 25 μ L real-time PCR using iQ™ SYBR Green Supermix catalog number 170-8882 (Bio-Rad, Hercules, CA) with an Mx3000P instrument (Stratagene (Agilent), Cedar Creek, TX). The cycle conditions were: 5 min at 95° C followed by 40 cycles of incubation at 94° C for 30 s, then 60 to 62° C for 1 min and 72° C for 30 s. Table 2 provides the

sequences of the primers used for this study (Supplemental Info). The primers were validated by size of PCR products. The absence of non-specific products and primer-dimers was confirmed by gel electrophoresis before running each sample analysis. Results were analyzed using the software provided with the Stratagene Mx3000P QPCR system. Differences in mRNA expression were calculated using the $\Delta\Delta CT$ method (Livak and Schmittgen, 2001) normalizing to DcAC1 (actin, GenBank accession X17526) or DcS15a (cytoplasmic ribosomal protein S15a, GenBank accession number AF349962) expression (Zagon et al., 2010). The primer list with sequences and some primer efficiencies is in Table 2 (Supplemental Info). Primer efficiencies ranged from 112 to 87 %.

Results

Experiment

Chlorogenic acid (CGA) is one of the products resulting from phenylpropanoid biosynthesis in carrot roots (Figure 1). Wounding induces phenylpropanoid biosynthesis in multiple plant species (see references in Introduction). Recently we demonstrated for certain crops that UV-B treatment in combination with wounding further induced phenylpropanoid biosynthesis over the levels resulting from wounding alone and that carrot, from among all the crops we tested, was the most responsive (Du et al., 2012, Avena-Bustillos et al., 2012, Du et al., 2014). To further our characterization of the synergistic effect of UV treatment and wounding we elected to evaluate the induction of phenylpropanoid biosynthesis based on the gene expression levels and expanded our metabolite analysis to include ferulic, caffeic, and coumaric acids in addition to chlorogenic acid (CGA). We previously observed that ferulic, caffeic, and coumaric acid levels were induced by UV-B treatment, but did not quantitate them.

Results for the targeted metabolite analysis from a seven-day time course are shown below (Figure 2). As previously found (Avena-Bustillos et al., 2012), CGA was the predominate peak in each of

the chromatograms (not shown). CGA concentrations ranged from 17.8 µg /g FW at day zero to as high as 1292.7 µg/g FW (Day 7, Sample A) and 314.8 µg/g FW (Day 6, Sample A) for treated and untreated samples, respectively. The rate of CGA accumulation was linear in the treated samples over the seven-day study period and up to day six in untreated samples. By day seven, the average CGA concentration in the treated carrots was approximately six times greater than the concentration found in untreated samples and 55 times greater than the concentration at day zero. UV-treatment also simulated the accumulation of caffeic, coumaric, and ferulic acids. Caffeic acid showed an increase over seven days, whereas ferulic and coumaric acids exhibited rapid rises in concentration. However the increase in coumaric acid quickly reversed itself, but in the case of ferulic acid, the elevated concentration persisted. In the untreated samples, ferulic acid accumulation was similar to that observed for treated samples, except the concentration was slightly less. Extractions were also tested for anthocyanin content using the pH difference method. No anthocyanins were detected in any of the samples (data not shown).

Gene Expression analysis

We examined expression profiles of the genes involved in CGA biosynthesis using real-time PCR. Many of the genes in this pathway have already been cloned from carrot (Table 1). However, some, such as *HQT* and *C3H* have been cloned from other plant species but not yet from carrot. To do a complete pathway analysis we applied same methodology we used successfully previously with a tomato EST database (Bartley and Ishida, 2003). In short, we performed BLAST search analyses of a carrot EST database assembly created by USDA/ARS researcher Dr. Philipp W. Simon (Altschul et al., 1997; Iorizzo et al., 2011; Yildiz et al., 2013) using the gene sequences for other plants. Iorizzo et al., 2011, identified a putative *PAL2*, *C4H*, and *4CL1* and 2 transcripts based on similarity with known genes. Xu et al., 2014 identified a number of anthocyanin biosynthesis pathway genes

including, *C4H*, *4CL*, and *CHI* in a study describing the differences between purple and non-purple carrots. We have identified putative transcripts (contigs) for HQT1, C3H1 and 2, HY5, and UVR8 based on similarity to genes from artichoke (*Cynara cardunculus*) and coffee (*Coffea canephora*) (Table 1).

Carrot slices were exposed to UV-B and total RNA was isolated at 0 h T₀ and T₁ and 2 h; 1, 2, 3 and 6 days with untreated slices used for comparison. Real-time PCR was performed to detect relative expression levels at each time point in treated or untreated slices. Relative expression calculations were performed by comparing time points 2 h to 6 days to the 0 h T₁ expression.

Phenylpropanoid Core Pathway

The PAL enzyme converts phenylalanine to cinnamic acid in the 1st step of the phenylpropanoid core pathway (Figure 1). There are three known, *DcPAL1*, 3, 4 and one putative, *DcPAL2* (contig 21482), *PAL* genes in carrot (Iorizzo et al., 2011). Our examination revealed a similar expression profile for all four with *PAL1* showing the greatest induction although this is not absolute expression (Figure 3A). All four *PAL* genes had a substantial basal expression of 24 to 26.5 cycles (threshold cycle Ct) with 40 cycles being the lowest possible in this PCR. In fact, most of these genes with the exception of *DcCHS2* had some basal level of expression even in untreated carrot slice at 0 h T₁. All 4 *PAL* genes were induced by UV-B treatment at least 4-fold by day 1 followed by a decrease at day 2 and returning nearly to steady state by day 6 (Figure 3A). *PAL1* was slightly different from the other *PAL* genes in that its expression level dropped near steady state level at day 3. Interestingly, in the untreated slices all the *PAL* genes increased somewhat at 2 h, then dropped below (*PAL1* and 2) or near (*PAL3* and 4) steady-state levels returning to the steady-state level on day 6 except for *PAL1*, which never returned to the initial level (Figure 3B). This induction at 2 h could be an indicator of a wound-induced component produced by the carrot slicing. *C4H*, the next step in the pathway

converts cinnamic acid to 4-coumaric acid. Steady state for *DcC4H1* was 23 cycles which increased 5-fold at 2 days and thereafter decreasing at 3 and 6 days (Figure 4). *4CL* converts 4-coumaric acid to 4-coumaroyl-CoA, an important precursor for many compounds such as CGAs, isoflavones, glycoflavones, flavonols, anthocyanins, lignins and tannins (Figure 1). The *Dc4CL* (combined) expression profile was much different than the other structural biosynthetic genes in that it revealed a huge induction at day 1 of almost 30-fold and decreasing to 3.7-fold on day 2, 3.8-fold on day 3 and 2.6-fold at day 6. Interestingly, in untreated slices, *Dc4CL* expression increased 3.3-fold at 2 h, decreasing to steady state on days 1 to 3, returning to 3.1-fold at day 6.

Chlorogenic acid biosynthesis

Two or three enzymes appear to be involved in the conversion of 4-coumaroyl-CoA to CGA (Lallemand et al., 2012; Mahesh et al., 2007; Sonnante et al., 2010). However, there is some debate over substrate specificities and principal routes (see discussion). HCT and HQT act either independently or in concert to convert 4-coumaroyl-CoA to 4-coumaroyl quinic acid and the meta-hydroxylase, C3H, converting 4-coumaroyl quinic acid to caffeoyl quinic acid (chlorogenic acid) (Sonnante et al., 2010). Alternative routes have been proposed (see discussion). *DcHCT1* expression was similar to *DcPAL1* in treated slices but peaked near 4.3-fold on day 2, decreasing to 2-fold on day 3 and returning to steady state by day 6. Untreated slices were quite different however as expression increased to 2.4-fold at 2 h and 2.3-fold by day 1 and decreasing to steady state on days 2 and 3 and increasing 3.1-fold on day 6 (Figure 5). Through our “BLAST” searches we found two different contigs similar to *Cynara cardunculus* CcHQT1 (GenBank Accession, EU697935) (Table 1). Both contigs, 42299 and 13256, were more similar to CcHQT1 (85 % similarity) than to CcHQT2 (GenBank Accession EU839580), 81 and 80 % similarity respectively. Therefore we decided to name them DcHQT1-1 and DcHQT1-2 respectively. *DcHQT1-1* and *DcC3H1* expression

profiles in treated slices are quite similar to each other and somewhat similar to *DcPAL1*, however their expression profiles in untreated slices although similar to each other, are quite different from *DcPAL1*. In treated slices, expression of both genes increases steadily to peak at approximately 9-fold on day 3 and decreasing on day 6 to 3.3-fold (*DcHQT1-1*) and 5.5-fold (*C3H1*) (Figure 5). Expression of *DcHQT1-1* and *DcC3H1* in untreated slices increased slightly above steady state of 25 and 27 cycles for *DcHQT1-1* and *DcC3H1* respectively from 2 h through day 3 and increased to about 4-fold on day 6.

Flavonoid Biosynthesis

Chalcone synthase (CHS) is the first key step directing substrates into the flavonoid branch of phenylpropanoid biosynthesis. CHS takes three molecules of malonyl CoA and one of 4-coumaroyl CoA and produces tetrahydrochalcone (chalcone). Products of this branch include anthocyanins, flavonols, glycoflavones and tannins. Carrot chalcone synthases were cloned and identified by Hirner and Seitz (2000) and Ozeki et al., (1993) from cell culture lines. We designed primers for *DcCHS1* and *DcCHS2* and performed real-time PCR to detect *DcCHS2* transcripts based on its strong expression in treated slices. *DcCHS1* and *DcCHS2* were the only genes we investigated that had no transcripts (>40 cycles) in the 0 h T₁ samples. Where there was no transcript (no Ct), we substituted 40 cycles in our delta-delta Ct calculations giving us rather large numbers for induction (Figure 6). We did still have large differences between treated and untreated slices for expression of *DcCHS2*. Treated slices showed an induction of *DcCHS2* of 216-fold at 2 h increasing to 5883-fold at 1 day, falling to 1742-fold at 2 days, increasing again at day 3 to 3042-fold and finally at 6 days greater than 20,000-fold. In untreated slices, *DcCHS2* increased to 32.4-fold at 2h and held at 38-fold until day 3 when it reached 155.6-fold and finally over 1200 -fold at day 6. *DcCHS1* had similar profiles as *DcCHS2* however expression was very low, the lowest cycle number was ~29 where the

lowest cycle number for *DcCHS2* was ~ 25 and there was little difference between treated and untreated except at 2h (data not shown). Chalcone isomerase (CHI) converts tetrahydroxychalcone to narigenin. Primers based on *DcCHI*, accession KM359964, (Table 1) were used to show expression of carrot *DcCHI* decreased at 2 h in treated slices and increased from 2-fold on day 1 to 7.1-fold on day 6. Untreated slices also showed an initial decrease with a following increase to 11-fold on day 6 (Figure 7).

Regulatory genes

We investigated various transcription factors known for involvement in stress and UV-B response (*DcMYB1*, *DcHY5*) or association with the *PAL* or *CHS* promoters (*DcPcMYB1*, *DcMYB3-2*, *DcMYB5*, *DcERF2*) (Maeda et al., 2005; Wako et al., 2010, Kimura et al., 2008; Feldbrügge et al., 1997; Ulm et al., 2004). *DcMYB1* is an important regulator of *PAL1* (Maeda et al., 2005). Expression of *DcMYB1* in treated slices fell below a steady state of about 26 cycles at 2 hrs and gradually increased to ~4 fold on day 3 holding there through day 6. *DcMYB1* expression in untreated slices also showed an initial decrease and stayed below steady state through day 6. *DcMYB3-2* and *DcMYB5* and *DcHY5* all showed great induction at 2 h, 16.5 to over 51.4-fold in treated but less, 2.9 to 20-fold, in untreated slices and all decreased to near or below steady state from day 1 to day 6 (Figures 8, 9 and Supplemental Figure 12). *DcPcMYB1* is the putative carrot homolog to the parsley (*Petroselinum crispum*) *MYB1*. *PcMYB1* was shown to be associated with the parsley *CHS* promoter sequence MRE^{CHS} involved in light response (Feldbrügge et al., 1997). Compared to the other genes, *DcPcMYB1* showed little change in our study (Figure 10). *DcERF2*, an ethylene response factor (ERF) family transcription factor that binds to a GCC-Box homolog in the *DcPAL3* promoter, was not induced (Kimura et al., 2008)(data not shown).

UV receptor

We also investigated the carrot homolog of UVR8, the UV receptor in *Arabidopsis* even though it is not transcriptionally regulated during the UV-B response (Tohge et al., 2011). UVR8 in *Arabidopsis*, is the UV-B receptor that interacts with AtCOP1, a WD40 domain containing E3 ubiquitin ligase, to stabilize and induce transcription of *AtHY5*, a bZIP transcription factor that induces UV response genes (Rizzini et al., 2011; Christie et al., 2012, Heijde and Ulm, 2012). We found two contigs quite similar to the *Arabidopsis thaliana* UVR8, Contigs 30663 (DcUVR8-1) and 8550 (DcUVR8-2) (Table 1). The predicted peptide sequences of these contigs contain the conserved tryptophans and arginines, required for UV-B reception (Christie et al., 2012). In our study, putative *DcUVR8-2* was more highly induced in untreated slices (Figure 9). However, the principal method for UVR8/COP1 transcriptional activation of Hy5 is post-translational (nuclear localization and COP1 interaction).

Discussion

Carrot roots are efficient producers of CGA and can be induced to produce higher amounts by exposure to UV-B light or wounding. In carrot, phenylpropanoid production was investigated primarily through studies of *PAL* gene expression and enzyme activity (Hirner et al., 2001; Ozeki et al., 2000). Hirner et al., 2001, and Ozeki and Kamamine, 1981, developed carrot cell culture lines to study anthocyanin production. These cell lines were examined at the gene expression level during UV-A exposure. *DcPAL1*, *DcCHS1* and 2, and other genes more specific to anthocyanin biosynthesis were induced by UV-A in a cell line that accumulated anthocyanins (Hirner et al., 2001). Ozeki et al., 2000, cloned genomic fragments for 3 *PAL* genes, g(genomic)*DcPAL1*, 3, and 4. *gDcPAL2* was revealed earlier to be *gDcPAL1* with a transposon that had been inserted during cell subculturing as it did not exist in wild-type carrot (Ozeki et al., 1997). *DcPAL1* was induced by UV-

B, elicitor, and dilution in experiments using carrot cell suspension cultures (Maeda et al., 2005). Also in this study *DcMYB1*, a MYB family transcription factor, was shown to be important for the expression of *DcPAL1*. *DcPAL1* was induced 1 h after *DcMYB1* rise in expression in all stress treatments. Interestingly, *DcMYB1* revealed a high peak of expression approximately 0.5 h after treatment (Maeda et al., 2005). We did not see this high of an early peak for *DcMYB1* in our experiment, but we did observe high early peaks of expression for *DcMYB3-2*, *DcMYB5* and *DcHY5*. *DcMYB3-2* and *DcMYB5* are also MYB family transcription factors and were shown to be involved with *DcPAL3* transcriptional activation (Wako et al., 2010). In the previous study, it is possible that because they were using full-length cDNA probes for Northern blots, that they may have also detected *DcMYB5* and *DcMYB3-1* and 2. There is a conserved MYB sequence at the 5 prime coding end of these MYB transcripts that may cross hybridize with the same sequence in *DcMYB1* (Figure 11). Our PCR analysis is specific for each transcript, with exceptions noted, avoiding this problem. In our study, we found some induction of expression for all four PAL genes including the newly identified putative *DcPAL2*. In untreated slices only *DcPAL3* showed an increase indicating that *DcPAL3* is induced by wounding. *DcMYB1* cannot be the only regulator of these genes however, because it initially decreases at 2 hrs after treatment and rebounds to over 2 fold higher than steady-state at 1 day (Figure 10). *AtHY5* is a bZIP transcription factor known to be involved with UV-B response in *Arabidopsis* in cooperation with *AtUVR8*, the UV-B photoreceptor and *AtCOP1*, another regulatory protein (Ulm et al., 2004; Christie et al., 2012). Speculatively, *DcMYB3-2* and *DcMYB5* or *DcHY5* may be responsible for the induction of the PAL genes at 2 h. However, chlorogenic acid biosynthesis has not been studied in carrot beyond the PAL enzyme. This is exemplified in that our group, Iorizzo et al., 2011 and Xu et al., 2014, have only recently identified cDNAs for the carrot genes involved in CGA biosynthesis e.g. *DcC4H1* and 2, *Dc4CL1* and 2,

DcHCT1, *DcHQT1* and *DcC3H1* and 2. These genes have been studied extensively in artichoke and coffee (Lepelley et al., 2007, Sonnante et al., 2010). In this study *DcC4H1* expression is similar to *DcPAL1* but peaks at 2 days about 5-fold higher than the steady state of 23 cycles (Figure 4). Similarity of the expression of these two genes is to be expected as they may form a “metabolon”, a channeled enzyme complex on the endoplasmic reticulum (ER) (Rasmussen and Dixon, 1999; Achnine et al., 2004). Enzyme complexes are thought to form for each of the possible final products coming from the phenyl propanoid pathway e.g. lignins, flavanols, anthocyanins, and isoflavonoids (Winkel, 2004; Jørgensen et al., 2005). These complexes are anchored to the ER by the various integral P450 membrane proteins in the complex like C4H, C3H, F3H, F5H, and IFS. *Dc4CL* expression is similar to *DcMYB3-2*, *DcMYB5* and putative *DcHY5* but with a peak at day 1 of ~30-fold and decreasing to about 4-fold over steady state thereafter (Figure 4). We should note here that our primers should amplify both *Dc4CL* transcripts identified by Iorizzo et al., 2011 and Xu et al., 2014, resulting in a combined profile for *Dc4CL*. *Arabidopsis* has at least three 4CLs each with different substrate preferences and expression profiles with At4CL3 probably participating in flavonoid production and all respond to UV-B exposure (Ehlting et al., 1999).

There are still some uncertainties as to the exact nature of the last steps in the route to CGA (Figure 1). A number of possible routes and enzymes exist to convert 4-coumaroyl-CoA to chlorogenic acid (Sonnante et al., 2010). *DcHCT* and *DcHQT* are quite similar with *DcHQT1-1* (contig42299) peptide sequence 87 % similar to *DcHQT1-2* (contig13256) and 76 % similar to *DcHCT* (contig19605). The difference lies in their preference for either quinic acid or shikimic acid. In coffee HQT prefers quinic and HCT prefers shikimic acid, however HCT is still capable of producing 3,5-O-dicaffeoylquinic acid ester, indicating a certain amount of flexibility in substrate specificity (Lallemand et al., 2012). *Ruta graveolens* C3H (CYP98A22) is more efficient at using 4-

coumaroyl quinate than 4-coumaroyl shikimate, but C3H (CYP98A3) prefers 4-coumaroyl shikimate (Karamat et al., 2012). It is unknown if this species contains HQT but it does contain HCT. Is it possible that HCT in this species is flexible enough to use both substrates? To investigate this further we performed a blast search of citrus, also a member of the rue family, ESTs using HQT1 from artichoke (*Cynara cardunculus*). We found only sequences that were most likely HCT when they were blasted back through the nonredundant (NR) GenBank database. Perhaps HCT can perform both functions in the rue family. In artichoke the principal route to CGA is believed to be $HCT \Rightarrow C3H \Rightarrow CGA$, but in coffee route 2, $HCT/HQT \Rightarrow C3H \Rightarrow CGA$, is thought to be the principal route (Lepelley et al., 2007; Mahesh et al., 2007; Comino et al., 2009; Sonnante et al., 2010; Karamat et al., 2012; Tohge et al., 2013). Silencing of *HQT* in potato recently showed the importance of *HQT* in CGA accumulation in leaves and tubers (Payyavula et al., 2015). Our real-time PCR results indicate that *DcHCT* has a similar expression profile as *DcC4H* and *DcPAL1* in treated slices, but *DcHQT1-1* and *DcC3H1* have strikingly similar profiles that are different from *DcPAL1* or *DcC4H* perhaps suggesting slightly different regulation. It is probable that some of these enzymes are found in more than one metabolon or complex. Such similar co-expression has been used in other investigations to suggest function in the same biosynthetic pathway (“guilt by association”) (Tohge and Fernie 2012; Gillis and Pavlidis 2012).

We also looked at two early steps in the flavonol and anthocyanin branch of the phenylpropanoid biosynthetic pathway, CHS and CHI. Two CHS genes, *DcCHS1* and *DcCHS2* are known to be induced by UV-A exposure in carrot (Hirner and Seitz 2000; Hirner et al., 2001). We found in preliminary PCR that *DcCHS2* appeared to be highly expressed in treated slices but *DcCHS1* was not although the two different primer sets cannot be directly compared (data not shown). Using real-time PCR we found that *DcCHS2* was greatly induced from a steady state of

none (40 cycles) to over 6000 fold (~25cycles) at 1 day in treated slices. Wounding also had an effect as the untreated slices increased 38 fold at 1 day. Hirner and Seitz, 2000, also found high induction of *DcCHS2* with somewhat less induction of *DcCHS1* after exposure of anthocyanin producing carrot cell cultures to UV-A. In one carrot cell line that did not produce anthocyanins after UV-A exposure, they found that only *DcCHS2* was induced. Initially, they found that the CHS in that cell line may be inhibited by malonyl-3'-dephospho-CoA produced by a 3'-Nucleotidase (Hinderer and Seitz, 1986). Later Hirner et al., 2001 suggested that anthocyanin production was dependent on DcCHS1 even though both DcCHS enzymes had catalytic activity. We would expand that to indicate that one possibility is that the complex or metabolon may be missing a key component in the non-anthocyanin producing line or the root tissue is missing other components present in leaves but not roots. *DcCHI* was a much different story as it was already expressed at 28 cycles "steady state" in controls and actually decreased at 2 h and returned to approximately steady state at 1 day and increasing to 7-fold by day 6. *DcCHI* may not need strict transcriptional control as narigenin chalcone can convert to narigenin spontaneously but the alfalfa CHI does direct the product formation toward the (2S) configuration that is better for ring closure (Jez and Noel, 2002).

In *Arabidopsis* leaves, response to UV-B exposure is mediated by the UV-B receptor, UVR8 (UV RESISTANCE LOCUS 8), the bZIP transcription factor HY5 (ELONGATED HYPOCTYL 5) and COP1 (CONSTITUTIVELY PHOTOMORPHOGENIC 1) with downstream transcription factors (MYBs) (reviewed in Heijde and Ulm, 2012). The mechanism for UV-B reception and signal transduction was recently reported by Christie et al., 2012 and Rizzini et al., 2011. UVR8 exists as a dimer held together by salt-bridges formed by a network of tryptophans in association with arginines. Exposure to UV-B light causes charge neutralization and dissociation of the dimer. UVR8 monomers then bind COP1 proteins and stabilize them activating HY5 gene expression

(Favory et al., 2009). HY5 is involved in many processes in the plant, primarily photomorphogenesis, but is also required along with UVR8 and COP1 for the UV-B response (Oravecz et al., 2006). HY5 can bind the *AtCHS* promoter and indeed binding sites were found in *AtPAL1*, *At4CL3* and *AtC4H* genes (Lee et al., 2007; Zhang et al., 2011). We did locate one canonical HY5 binding site (CACGTG, Lee et al., 2007) approximately 70 nt upstream of the transcription start site of *DcCHS2*. HY5 regulates flavonoid biosynthesis in *Arabidopsis* through the downstream transcription factors, AtMYB4, AtMYB11, AtMYB12, AtMYB111 and PAP1/AtMYB75 (Stracke et al., 2007). These MYB TFs activate gene expression of biosynthetic genes such as, *CHS*, *CHI*, *F3H* and flavonol synthase (Favory et al., 2009). Accumulation of flavonols does not occur in the *Atmyb11*, *Atmyb12*, *Atmyb111* triple mutant of *Arabidopsis* but there is still a UV-B response, indicating still other regulators involved in this process (Stracke et al., 2010). *AtMYB12* over expression in transgenic tomato plants causes an increased flavonol and CGA accumulation (Luo et al., 2008). Hierarchical cluster analysis of *Arabidopsis* UV-B exposure experiments contained in the ATTED-II database indicated very early induction of *AtHY5* (15 min) followed by *AtMYB12* and *AtMYB111* (30 min to 1 h) induction and finally *AtCHS* and *AtC4H* at 3 h but no change in UVR8 expression (Tohge et al., 2011). This result for *DcUVR8* expression is expected because it is a receptor and its principal action is post-translational de-dimerization and nuclear localization. Additionally, it has recently been shown that another, unidentified receptor, possibly containing a tetrahydrobiopterin ligand may also be involved in UV-B induction of anthocyanin production (Takeda et al., 2014). Thus, it is not surprising that in our experiment UV-B exposure first induces *DcHY5*, *DcMYB3-2* and *DcMYB5* followed by induction of *DcMYB1* and the downstream biosynthetic genes *DcPAL1-4*, *DcC4H*, *DcC4L*, *DcHCT*, *DcHQT1-1*, *DcC3H1* and

DcCHS2. Our results indicate possible cooperative action of *DcHY5* and the various MYBs to initiate and sustain a regulatory repertoire for production of CGA.

Conclusion

In this experiment the expression of a number of the genes involved in phenylpropanoid biosynthesis following UV-B exposure of carrot slices has been shown. Genes in the core pathway including *PAL1-4*, *C4H1*, *4CL1* and *4CL2*(combined), *CH11*, and *CHS1* and *CHS2* were investigated. We have also identified putative transcripts for *DcHQT1*, *DcC3H1* and *DcC3H2*, *DcHY5* and *DcUVR8* and analyzed their expression using real-time PCR. More importantly, we have analyzed a number of known regulatory TFs including *DcHY5* and found that their expression correlates with induction of the phenylpropanoid biosynthetic pathway. It is quite interesting to find that the UVR8 – HY5 signaling network appears to be functioning in carrot roots. Hopefully, our results will stimulate further research in this area.

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Figure 1.

Figure 2.

Figure 3A.

Figure 3B.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Figure 9.

Figure 10.

DcMYB1	142	AGGTGTGGGAAGAGTTGCAGGCTGAGATGGATCAACTATTTGCGACCAGATAT	194
DcMYB5	216	AGGTGCGGGAAGAGCTGCAGGCTGAGATGGCTCAACTATTTGAGACCGGATAT	268
DcMYB3-2	147	AGATGTGGAAAGAGTTGCAGGCTGAGATGGTTGAATTATTTGAGACCGGATAT	199
DcMYB3-1	147	AGATGCGGAAAGAGTTGCAGGCTGAGATGGTTGAATTATTTGAGACCGGATAT	199

Figure 11.

Figure Legends.

Figure 1. Core phenylpropanoid biosynthetic pathway illustrating important steps analyzed in this study. PAL: phenylalanine ammonia-lyase, C4H: trans-cinnamate 4-monooxygenase, 4CL: 4-coumarate: CoA ligase, HCT: hydroxycinnamoyl-CoA: shikimate/quinic acid hydroxycinnamoyltransferase, HQT: hydroxycinnamoyl CoA quinate transferase, and C3H: 4-coumarate 3-hydroxylase CHS: Chalcone synthase, CHI: Chalcone isomerase

Figure 2. Phenolic acids determined by HPLC for three independent samples (A, B, C). Dashed lines were used for treated samples. Results reported in ng/g fresh weight.

Figure 3. RT-PCR expression analysis of *DcPAL1-4* after treatment with UV-B light. A. Expression of *DcPAL1-4* in treated carrot slices. B. Expression of *DcPAL1-4* in untreated carrot slices.

Figure 4. RT-PCR expression analysis of *DcC4H1* and *Dc4CL1* in treated and untreated carrot slices.

Figure 5. RT-PCR expression analysis of *DcHCT1*, *DcHQT1-1* and *DcC3H1* in treated and untreated carrot slices.

Figure 6. RT-PCR expression analysis of *Dc4CHS2* in treated and untreated carrot slices.

Figure 7. RT-PCR expression analysis of *DcCHI* treated and untreated carrot slices.

Figure 8. RT-PCR expression analysis of *DcMYB3-2* and *DcMYB5* in treated and untreated carrot slices.

Figure 9. RT-PCR expression analysis of *DcHY5* and *DcUV8-2* in treated and untreated carrot slices.

Figure 10. RT-PCR expression analysis of *DcMYB1* and *DcPcMYB1* in treated and untreated carrot slices.

Figure 11. Alignment of *DcMYB1* (GenBank ID:AB218778), *DcMYB5* (GenBank ID:AB298509), *DcMYB3-2* (GenBank ID:AB298507), and *DcMYB3-1* (GenBank ID:AB298506), showing conserved nucleotides.

Table 1.
Phenylpropanoid biosynthesis enzymes and Regulators

Protein	organism	Gene Symbol	GenBank ID	Sequence used for BLAST search	Carrot Contig ID	Percent similarity	Expression after treatment	References
Phenylpropanoid biosynthesis enzymes								
Phenylalanine ammonia lyase	<i>Daucus carota</i>	PAL1	D85850	* 1-708	Contig21709	99	++	Ozeki and Takeda, 1994; Iorizzo et al., 2011
	<i>Petroselinum crispum</i>	PAL2	X81158	* 1-716	Contig21482	97	++	Iorizzo et al., 2011
	<i>Daucus carota</i>	PAL3	AB089813		NA		++	Ozeki and Takeda, 1994;
	<i>Daucus carota</i>	PAL4	AB435640	* 1-715	Contig28113	100	++	Ozeki et al., 2000; Iorizzo et al., 2011
cinnamate 4-hydroxylase	<i>Daucus carota</i>	C4H1	KM359961	#1-1798	Contig524	99	++	Xu et al., 2014, Iorizzo et al., 2011
trans-cinnamate 4-monoxygenase	<i>Daucus carota</i>	C4H2	KM359962				not done	Xu et al., 2014
4-coumarate:CoA ligase	<i>Daucus carota</i>	4CL1	KM359963	#1-1814	Contig8294, Contig52163	97	++	Xu et al., 2014, Iorizzo et al., 2011
	<i>Daucus carota</i>	4CL2	KM359966	#1-1641	Contig32987	99	both amplified	Xu et al., 2014
hydroxycinnamoyl-CoA:quinase	<i>Daucus carota</i>	HCT1	KR559236	# 1-1290	Contig19605	99	++	Unpublished?
hydroxycinnamoyltransferase								
hydroxycinnamoyl CoA quinate transferase	<i>Cynara cardunculus</i>	HQT1	EU697935	* 18-449	Contig42299(HQT1-1)	85	++	this paper
					Contig13256(HQT1-2)	85	not done	this paper
p-coumaroyl 3'-hydroxylase	<i>Coffea canephora</i>	C3H1	DQ269126	* 1-508	Contig16387	88	++	this paper
	<i>Coffea canephora</i>	C3H2	DQ269127	* 17-507	Contig9460	91	not done	this paper
Flavonoid Biosynthesis enzymes								
chalcone synthase	<i>Daucus carota</i>	CHS1	AJ006779	# 11-1377	Contig16173	97	+	Hirner and Seitz, 2000
	<i>Daucus carota</i>	CHS2	AJ006780	# 16-1496	Contig45955	97	+++	Hirner and Seitz, 2000
chalcone isomerase	<i>Daucus carota</i>	CHI1	KM359964	#1-735	Contig16284	99	+	Xu et al., 2014
Transcription factors								
R2R3 MYB transcription factor	<i>Daucus carota</i>	DcMYB1	AB218778	# 101-702	Contig17550	98	++	Maeda et al., 2005
	<i>Daucus carota</i>	DcMYB3-1	AB298506	# 121-785	NA	98	not done	Wako et al., 2010
	<i>Daucus carota</i>	DcMYB3-2	AB298507	# 121-788	Contig10727	100	+++	Wako et al., 2010
	<i>Daucus carota</i>	DcMYB5	AB298509	# 362-901	Contigs 1165 and 29345	99	+++	Wako et al., 2010
MYB transcription factor	<i>Petroselinum crispum</i>	PcMYB1	U67133	* 9-307	Contig59299	79	little change	this paper
bZIP transcription factor	<i>Arabidopsis thaliana</i>	HY5	AB005295	* 28-148	Contig32104	78	+++	this paper
Ethylene response factor 1	<i>Daucus carota</i>	ERF1	AB354581	# 48-1120	Contig6449	99	not done	Kimura et al., 2008
Ethylene response factor 2	<i>Daucus carota</i>	ERF2	AB354582	# 11-1184	Contig595	95	--	Kimura et al., 2008
UV-B receptor								
UV-B resistance	<i>Arabidopsis thaliana</i>	UVR8-1	NM_125781	* 16-440	Contig30663 (UVR8-1)	88	not done	this paper
		UVR8-2			Contig8550 (UVR8-2)	86	+	this paper

* = amino acid sequence

= nucleotide sequence

Supplemental Table 2.

Gene	Primer Name	size(bp)	Primer pair	Primer Sequence (5'-3')	% Efficiency
<i>DcPAL1</i>	21709F1	331	Forward	CTCTGCAGAAGGAGCTTATTAGATT	89.3
	DCPAL1R1		Reverse	GCTTCTGCAGCAGTCAGATTC	
<i>DcPAL2</i>	21482F1	325	Forward	CTCTTCAGAAAGAGCTCATTAGGTT	99.6
	21482R1		Reverse	GCTTCTTCGGGGCTGAGAACGG	
<i>DcPAL3</i>	DCPAL34F1	193	Forward	CTCTTCAGAAGGAGCTCATTAGGTT	90.4
	DCPAL3R1		Reverse	ATGTTGTGGTTAAGAACTTGGTGA	
<i>DcPAL4</i>	DCPAL34F1	193	Forward	CTCTTCAGAAGGAGCTCATTAGGTT	
	DCPAL4R1		Reverse	ATGTTGTGGTTAAGAACTTGGTAA	
<i>DcC4H</i>	DCC4HF1	238	Forward	AGTTTAGGCCAGAGAGGTTCTT	97.5
	DCC4HR1		Reverse	TTCAAAATGTGTAGACTGAACTGT	
<i>Dc4CL</i>	DCC4CLF1	243	Forward	CGAGATCAAATCGGGTGC	92.3
	DCC4CLR1		Reverse	TCAACAATAAATAGCTCATCATC	
<i>DcHCT</i>	DCCHCTF1	265	Forward	CTACTCTCTTTTGGTATTGCAGGT	

	DCCHCTR1		Reverse	AGGAACTGCATCAGGACTGGAC	
<i>DcHQT1-1</i>	DCCHQT1-1F1	270	Forward	TGACTCACGTACTGTCAAAGAAG	105.4
	DCCHQT1-1R1		Reverse	TTGAACCGAGTCACCTGTGCA	
<i>DcC3H1</i>	DCC3H1F1	202	Forward	CAGACCGGATTATGACTGAGGCT	87.2
	DCC3H1R1		Reverse	AATGCAGGGTCACGGGCAATA	
<i>DcPcMYB1</i>	DCPCMYB1F1	270	Forward	CCAACATTGATTAAAGGATAAGTG	
	DCPCMYB1R1		Reverse	CTTCAAAAATCAAATTGGAGTACCTT	
<i>DcMYB1</i>	DCMYB1F1	267	Forward	GAGGGCTCTTCCTAAACTAGCTG	98.6
	DCMYB1R2		Reverse	GACTCATTTTTGACCTGATCATAG	
<i>DcMYB3-2</i>	DCMYB32F1	221	Forward	CCATTGGTTCAACCAGCATTAC	96.7
	DCMYB32R1		Reverse	TTCCAAAACCTCCTCCAGTATCT	
<i>DcMYB5</i>	DCMYB5F1	238	Forward	CCAAATCAGATCACCATCCCATA	93.8
	DCMYB5R1		Reverse	GAAATGAGTGAAGTAGTTTCAATCTTAG	
<i>DcERF2</i>	DCERF2F1	305	Forward	CGACAACATTTTCAGCCTCCTAA	88.4
	DCERF2R1		Reverse	ACACATGAAGCTGATCCTGACA	
<i>DcHY5</i>	DCHY5F1	141	Forward	GGCTGACAAAGAGAATAAGCG	

	DCHY5R1		d Reverse	TCAAGCTCAGAGTTTTTCTTCTC	
<i>DcUVR8-2(b)</i>	DCUVR8bF1	256	Forward	ACTGATAAACAGAATGTATACTCGT	
	DCUVR8bR1		Reverse	TTGTAATATCCTTTGCTGCGGG	
<i>DcCHS2</i>	DCCHS2F1	257	Forward	CTCAAATTCAAGCGAATGTGTGAAA	88.1
	DCCHS2R1		Reverse	CCGGGCATGTCTACACCAGAA	
<i>DcCHS1</i>	DCCHS1F1	256	Forward	GAGAAGTTTAGGCGGATGTGTGAA A	
	DCCHS1R1		Reverse	CAGGCATATCGACACCGCT	92.3
<i>DcCHI</i>	DCCHIF1	214	Forward	CCACGATTTTGCCATTAAGT	
	DCCHIR1		Reverse	TCCTTGGAGAAGCCGATGGTCAAT	
<i>DcS15A</i>	DCS15AF1	208	Forward	GTCATGCAAAAGCATGGTTACAT	92.3
	DCS15AR1		Reverse	CAGCAGATGTAGTCAGCACGATAT AA	
<i>DcACTAc1</i>	DCACTF1	140	Forward	AAAGGATGCCTATGTTGGT *	112.5
	DCACTR1		Reverse	CACGCAGCTCATTGTAGAA *	
<i>DcACTAc1</i>	DCACTF2	212	Forward	TCACACTTTCTACAATGAGCTG	98.5
	DCACTR4		Reverse	CAGAGTCCAAAACAATACCTG	

* - Primer sequence from reference (Zagon et al., 2010)

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