The tomato carotenoid cleavage dioxygenase 1 genes contribute to the formation of the flavor volatiles β-ionone, pseudoionone, and geranylacetone

Andrew J. Simkin1, Steven H. Schwartz2, Michele Auldridge1, Mark G. Taylor1 and Harry J. Klee1,*

1Horticultural Sciences, Plant Molecular and Cellular Biology Program, PO Box 110690, University of Florida, Gainesville, FL 32611-0690, USA and
2Department of Energy-Plant Research Laboratory and Department of Plant Biology, Michigan State University, East Lansing, MI 48824, USA

Received 17 April 2004; revised 18 August 2004; accepted 6 September 2004.

*For correspondence (fax +001 352 846 2063; e-mail hjklee@ifas.ufl.edu).

Summary

Volatile terpenoid compounds, potentially derived from carotenoids, are important components of flavor and aroma in many fruits, vegetables and ornamentals. Despite their importance, little is known about the enzymes that generate these volatiles. The tomato genome contains two closely related genes potentially encoding carotenoid cleavage dioxygenases, LeCCD1A and LeCCD1B. A quantitative reverse transcriptase-polymerase chain reaction analysis revealed that one of these two genes, LeCCD1B, is highly expressed in ripening fruit (4 days post-breaker), where it constitutes 0.11% of total RNA. Unlike the related neoxanthin cleavage dioxygenases, import assays using pea chloroplasts showed that the LeCCD1 proteins are not plastid-localized. The biochemical functions of the LeCCD1 proteins were determined by bacterial expression and in vitro assays, where it was shown that they symmetrically cleave multiple carotenoid substrates at the 9,10 (9',10') positions to produce a C14 dialdehyde and two C13 cyclohexones that vary depending on the substrate. The potential roles of the LeCCD1 genes in vivo were assessed in transgenic tomato plants constitutively expressing the LeCCD1B gene in reverse orientation. This over-expression of the antisense transcript led to 87–93% reductions in mRNA levels of both LeCCD1A and LeCCD1B in the leaves and fruits of selected lines. Transgenic plants exhibited no obvious morphological alterations. High-performance liquid chromatography analysis showed no significant modification in the carotenoid content of fruit tissue. However, volatile analysis showed a ≥50% decrease in β-ionone (a β-carotene-derived C13 cyclohexone) and a ≥60% decrease in geranylacetone (a C13 acyclic product likely derived from a lycopene precursor) in selected lines, implicating the LeCCD1 genes in the formation of these important flavor volatiles in vivo.

Keywords: fruit, apocarotenoid, taste, transgenic plants, aroma.

Introduction

Tomato fruits contain a complex mixture of volatile and non-volatile compounds that contribute to the overall aroma and taste of the fruit. The volatile aroma compounds are essential for good tomato flavor (Baldwin et al., 2000). These secondary metabolites are derived from a range of precursors including carotenoids, lipids, and amino acids. Despite their important roles, very little is known about the pathways for synthesis of many of these volatile compounds. Indeed, the biological functions of the volatile secondary products beyond their impact on flavor are in many cases not known. Of particular interest are a group of terpenoid flavor volatile compounds generally present at relatively low levels but possessing strong effects on the overall human appreciation of, for example, the flavor of tomato (Baldwin et al., 1991, 2000; Buttery et al., 1971, 1987), carrots (Kjeldsen et al., 2003), quince (Lutz and Winterhalter, 1992), and Averrhoa carambola (Winterhalter and Schreier, 1995). Among these are β-ionone, geranylacetone (6,10-dimethyl-5,9-undecadien-2-one) and pseudoionone (6,10-dimethyl-3,5,9-undecatrien-2-one). The biosynthetic routes leading...
to these C_{14} and C_{13} compounds have remained obscure. Based on their chemical structures and studies of volatile production in tomato varieties with unusual carotenoid accumulation, Buttery et al. (1988) predicted that these compounds are likely products of oxidative carotenoid cleavage. The potential relationships between these volatiles and their carotenoid precursors following 9,10 or 9',10' oxidative cleavage are shown in Figure 1. However, proof for the in vivo mechanisms for synthesis of these volatile compounds has heretofore been lacking.

In recent years, a family of carotenoid cleavage dioxygenases (CCDs) that cleave carotenoid substrates at a variety of double bonds have been identified. The first member of the family to be identified was VP14, a 9-cis-epoxycarotenoid dioxygenase involved in the synthesis of the phytohormone abscisic acid (Tan et al., 1997). Bouvier et al. (2003b) identified a zeaxanthin-specific 7,8 (7',8')-cleavage dioxygenase (CsZCD) from Crocus sativus encoding an enzyme capable of forming crocetin dialdehyde and 3-hydroxy-β-cyclitol in vitro. Crocetin dialdehyde is known to accumulate in the flowers of Jacquinia angustifolia (Eugster et al., 1969) and in the roots of Coleus forskohlii (Tandon et al., 1979). 3-hydroxy-β-cyclitol is believed to be the first committed step in the formation of safranal, a constituent of the spice saffron in C. sativus (Bouvier et al., 2003b). Bouvier et al. (2003a) have also identified a lycopene-specific 5,6 (5',6')-cleavage dioxygenase (BoLCD) from Bixa orellana, responsible for the formation of bixin dialdehyde and a C_7 cleavage product previously identified as 6-methyl-5-hept-2-one (MHO; Fay et al., 2003). MHO has been identified as an important contributor to tomato flavor (Baldwin et al., 2000; Buttery et al., 1990).

Recently a carotenoid cleavage dioxygenase, AtCCD1, that symmetrically cleaves the 9,10(9',10') double bonds of...
multiple carotenoid substrates into a C_{14} dialdehyde and two C_{13} cyclohexone derivatives in vitro has been identified (Schwartz et al., 2001). Orthologs of AtCCD1 have been found in a variety of species including Phaseolus vulgaris (Schwartz et al., 2001), Capsicum annuum (Bouvier et al., 2003b), C. sativus (Bouvier et al., 2003b) and Petunia hybrida (Simkin et al., 2004). As Schwartz et al. (2001) had previously shown that AtCCD1 cleaves a broad range of carotenoid substrates in vitro, we sought to determine whether an AtCCD1 homolog could be responsible for generating the C_{13} and C_{14} terpenoid flavor volatiles in tomato. Here, we show that the tomato CCD1 homologs contribute to synthesis of several important tomato volatile compounds that contribute to flavor.

Results

Identification of LeCCD1A and LeCCD1B in tomato

Based on the prior in vitro results of Schwartz et al. (2001), we hypothesized that enzymes homologous to AtCCD1 would be excellent candidates involved in generating volatiles such as geranylacetone and β-ionone. Therefore, we searched the available tomato EST database (http://www.tigr.org/tdb/lgi/) for sequences with significant homology to AtCCD1. We identified two different EST sequences with high nucleotide identity (74 and 72%) to Arabidopsis CCD1. The tomato CCDs each have open-reading frames of 1638 nucleotides. The predicted tomato CCD1 proteins are highly similar to the Arabidopsis (Schwartz et al., 2001) and petunia (Simkin et al., 2004) proteins (Table S1, Supplementary Material). Based on their high identity to AtCCD1 and to each other (83%), as well as their identical enzyme activities (see below), the tomato enzymes were designated as LeCCD1A (AY576001) and LeCCD1B (AY576002). Southern blot analysis confirmed that only two copies of LeCCD1 exist in the tomato genome (data not shown).

Activities of LeCCD1 proteins

A comparison of the predicted LeCCD1 proteins to the nine members of the Arabidopsis CCD family indicated the highest level of identity to AtCCD1, an enzyme that catalyzes the symmetrical 9-10(9’,10’) cleavage of a variety of carotenoids (Schwartz et al., 2001). To confirm the function of the LeCCD1 proteins, the genes were cloned into the pDEST15-GST fusion vector (Invitrogen, Carlsbad, CA, USA) for expression in Escherichia coli, and the purified recombinant proteins were assayed for cleavage activity on a variety of carotenoid substrates.

We first introduced plasmids expressing recombinant LeCCD1A and LeCCD1B proteins into strains of E. coli that were previously engineered to accumulate different carotenoid compounds (Cunningham et al., 1994, 1996; Sun et al., 1996). The carotenoids that accumulate in these strains impart color upon the cells and a loss of color indicates that the carotenoids are metabolized to colorless compounds. When each of the two recombinant proteins was expressed in cells producing β-carotene, lycopene, β-carotene, ε-carotene, δ-carotene, or zeaxanthin, there was less accumulation of pigment (Figure 2a). These results suggested that both enzymes could catabolize a range of linear and cyclic carotenoid substrates. However, loss of color resulting from catabolism of precursors to the accumulating carotenoids in each strain cannot be eliminated by this approach.

Assuming that the LeCCD1 enzymes have 9,10 (9’,10’) cleavage specificities, specific products would be generated, based on the carotenoid precursor that is catabolized (Figure 1). In order to precisely determine the activity and substrates of the LeCCD1 enzymes, purified recombinant LeCCD1A enzyme was assayed in vitro using multiple carotenoid substrates. The assay products were characterized by thin-layer chromatography (Figure 2b) and high-performance liquid chromatography (HPLC) (Figure 2c). In assays containing β-carotene, zeaxanthin, lutein, violaxanthin and neoxanthin, the central C_{14} dialdehyde (I) cleavage product was the major compound resulting from symmetrical cleavage at the 9,10 and 9’,10’ positions (Figure 2b). Although it is not apparent from the chromatogram in Figure 3(b), the predicted central cleavage product was detected by HPLC in reactions using lycopene and β-carotene as well as lutein (Figure 2c). Peak I on the chromatogram is the C_{14} dialdehyde. C_{15} products, resulting from cleavage at the 9,10 and the 9’,10’ positions, were also identified (Figure 2b). In assays containing violaxanthin or neoxanthin, 5’6-epoxy-3-hydroxy-β-ionone (II) was formed. Asymmetric cleavage also led to the formation of a C_{27} epoxy-apocarotenal (III) with these substrates. In assays containing neoxanthin, the asymmetric cleavage also led to the formation of a C_{27} allene-apocarotenal (IV) and the C_{13} grasshopper ketone (V). The C_{27} allene-apocarotenal and the C_{27} epoxy-apocarotenol can be subsequently recleaved making them difficult to see on thin layer chromatography (TLC) plates. Several of these characterized products are labeled on the TLC plate (Figure 2b). In assays containing lutein as substrate, symmetrical cleavage at the 9,10 and 9’,10’ positions led to the formation of both 3-hydroxy-β-ionone (VI) and 3-hydroxy-α-ionone (VII). Although the E. coli expression assays (Figure 2a) clearly indicated that the enzymes can cleave linear carotenoids, the in vitro purified enzyme did not work as effectively on these substrates as it did on the cyclic carotenoids (Figure 2b). Taken together, the E. coli expression and the in vitro data indicate that the tomato CCD1 enzymes, like the Arabidopsis CCD1, have broad substrate specificity.
Production of LeCCD1 plants with reduced expression

Although the results presented above are consistent with the in vitro results previously reported for AtCCD1, we sought to determine whether these enzymes are responsible for production of the major flavor and aroma volatiles in vivo. Therefore, transgenic tomato plants with reduced expression of LeCCD1A and LeCCD1B were produced and the effects of the reduced gene expression on the putative carotenoid-derived volatiles determined. As LeCCD1A and LeCCD1B are 83% identical, we chose to reduce the expression of both genes with a single constitutively expressed antisense construct pHK-LeCCD1 (see Experimental procedures) under the control of the constitutive Figwort Mosaic Virus (FMV) 35S promoter (Richins et al., 1987). Multiple independently derived plants transformed with the construct exhibited significant reductions in expression of the LeCCD1 genes. Gene expression in the transgenic lines varied from 7 to 90% of wild type levels. Two independent transgenic lines showing the greatest degree of gene silencing were selected for further study. Lines 1 and 2 showed 90 and 87% decreases in LeCCD1A transcript levels in leaves, respectively, and 88 and 93% decreases in LeCCD1B, respectively, in the same tissue (Figure 3). Similar decreases in LeCCD1 transcript levels were observed in fruits.

Five plants of each line and untransformed controls were grown for phenotypic characterization under standard greenhouse conditions. Analysis of time to first flower, first fruit and stem length indicated no significant differences between wild type and transgenic plants showing significant reduction in the expression of the LeCCD1 genes, under our experimental conditions (data not shown).

Effects of LeCCD1 gene silencing on volatile production

In order to determine the roles of LeCCD1 gene products in the formation of carotenoid-derived volatiles in vivo, volatile analysis was performed on ripe fruits. A total of 50–75 fruits from between four and seven plants were analyzed for each line. Fruits were staged by pericarp and jelly color upon opening. Lines 1 and 2 showed 37 and 45% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in ζ-carotene, respectively, and 88 and 93% decreases in LeCCD1B, respectively, in the same tissue (Figure 3). Similar decreases in LeCCD1 transcript levels were observed in fruits.

Five plants of each line and untransformed controls were grown for phenotypic characterization under standard greenhouse conditions. Analysis of time to first flower, first fruit and stem length indicated no significant differences between wild type and transgenic plants showing significant reduction in the expression of the LeCCD1 genes, under our experimental conditions (data not shown).

Effects of LeCCD1 gene silencing on volatile production

In order to determine the roles of LeCCD1 gene products in the formation of carotenoid-derived volatiles in vivo, volatile analysis was performed on ripe fruits. A total of 50–75 fruits from between four and seven plants were analyzed for each line. Fruits were staged by pericarp and jelly color upon opening. Lines 1 and 2 showed 37 and 45% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N). Furthermore, 56 and 61% decreases in β-ionone levels, respectively (Figure 4a), when compared with control plants (M82) and negative segregants for the transgene (N).
geranylacetone, a volatile potentially derived from phytoene, phytofluene or \( \beta \)-carotene, were also observed in lines 1 and 2, respectively (Figure 4b). Due to the fact that pseudoionone co-eluted with other volatiles in our system, we were unable to confirm effects of reduced gene expression on this lycopene cleavage product \textit{in vivo}. Carotenoid analysis in fruits from these lines indicated no significant alterations in phytoene, lycopene, \( \beta \)-carotene, or lutein content (data not shown). As a control for volatile emissions, the emissions of two unrelated volatiles were also determined (Figure 4c). There were no significant differences between transgenic and control lines for the leucine-derived volatile, 3-methylbutanol, nor the isoleucine-derived volatile, 2-methylbutanol.

LeCCD1A and LeCCD1B are expressed in all tissues

To quantify mRNA levels derived from each LeCCD1 gene, a transcript-specific assay based on fluorescent real-time reverse transcriptase-polymerase chain reaction (RT-PCR) (TaqMan; Applied Biosystems, Foster City, CA, USA) was developed. Absolute mRNA levels were quantified against a standard curve of tritium-labeled, \textit{in vitro}-transcribed sense strand RNAs. Gene-specific primers and probes were designed and tested for each of the two transcripts. Using TaqMan, expression of the \textit{LeCCD1} genes was quantified in leaves, roots, flowers, and fruits at various developmental stages (Figure 5). \textit{LeCCD1A} transcript was detected at a low level in leaves, representing 0.003% of total mRNA (Figure 5a). This contrasts to the level of \textit{LeCCD1B} in the leaves of the same plants, which was 13-fold higher (0.037% of total mRNA, Figure 5b). In roots, \textit{LeCCD1A} and \textit{LeCCD1B} were expressed at similar levels. \textit{LeCCD1A} represented 0.002% of mRNA in the fruit ovary. A decrease in transcript levels was observed throughout the ripening process until the red ripe stage (10 days post-breaker), where a twofold increase was observed. In contrast, \textit{LeCCD1B} transcript levels in the ovary were fourfold higher than \textit{LeCCD1A}.
levels and increased throughout the ripening process to a high of approximately 0.01% of mRNA in orange fruit (4 days post-breaker).

Localization assays for LeCCD1A and LeCCD1B

In Arabidopsis, all of the CCD proteins except CCD1 are predicted by computer modeling (TargetP, v1.1) to contain plastid transit peptides and have been shown to be taken up by chloroplasts (Tan et al., 2003; M. Auldridge and H.J. Klee, unpublished data). AtCCD1 is not imported into chloroplasts in vitro (Tan et al., 2003). Similarly, immunohistolocalization showed that a crocus CCD1 homolog is cytoplasmic (Bouvier et al., 2003b). Similar to AtCCD1, the deduced peptide sequences of LeCCD1A and LeCCD1B did not indicate the presence of plastid transit peptides (Table S1, Supplementary Material). To check for the presence of a unique plastid targeting sequence, in vitro-translated and tritium-labeled LeCCD1 proteins were tested for import into pea chloroplasts under conditions as described previously (Cline et al., 1993). Following import incubation, chloroplasts were purified, washed and treated with thermolysin to degrade proteins outside or adhering to the chloroplast outer envelope. The LeCCD1 proteins were sensitive to thermolysin treatment indicating that they were not imported into chloroplasts (Figure 6). However, after washing, partial binding of in vitro-translated LeCCD1 proteins to the surface of intact pea chloroplasts not treated with thermolysin was detected. Ribulose 1,5-bisphosphate carboxylase small subunit precursor (SSU) from Pisum sativum L. (Lamppa, 1988), and the light harvesting complex protein (LHCP) were used as positive controls for chloroplast uptake (Anderson and Smith, 1986). These results indicate that although the LeCCD1 proteins do have some affinity for the outer plastid membrane, they are not imported into chloroplasts.

Discussion

LeCCD1 enzyme activity in vitro and in vivo

The familiar ‘flavor’ of a fresh tomato is the consequence of a complex interaction between sugars, acids and a large number of volatile compounds synthesized during fruit ripening (Baldwin et al., 2000). These volatiles are believed to be derived from catabolism of a diverse set of precursors, including amino acids, fatty acids and carotenoids (reviewed in Buttery and Ling, 1993). Despite the importance of these volatiles to the flavor and aroma of many fruits, vegetables and ornamentals, in most cases the pathways and the enzymes for their synthesis are not known. We have been interested in the terpenoid volatiles such as \( \beta \)-ionone, pseudoionone and geranyl acetone that have been postulated to be products of oxidative cleavage of carotenoids. Because of their chemical structures, they have been assumed to be carotenoid-derived. This conclusion is supported by correlations with the abundance of certain volatiles with carotenoid composition in different tomato varieties (Buttery et al., 1988). Here, we have shown that LeCCD1A and LeCCD1B catalyze the symmetric cleavage of multiple cyclic carotenoids in vitro at the 9,10(9',10') double bonds resulting in the formation of a
variety of apocarotenoids, specific for the substrate. These observations are consistent with previously reported in vitro results for the Arabidopsis ortholog AtCCD1 (Schwartz et al., 2001) and the C. sativus homolog CsCCD (Bouvier et al., 2003b). We have further shown that LeCCD1A and LeCCD1B cleave lycopene, a C40 acyclic carotenoid, responsible for the red color in tomato fruit, into a C14 dialdehyde and pseudoionone. Due to the fact that pseudoionone is a recognized volatile component of tomato (Buttery et al., 1971) and from the in vivo analysis we conclude that CCD1 is a likely contributor to its formation in vivo.

We have further demonstrated, in vivo, that plants with reduced LeCCD1 expression show a significant decrease in the formation of geranylacetone, a C15 dialdehyde potentially derived from 9,10(9′,10′) cleavage of phytoene. Other possible substrates for the formation of geranylacetone include phytofluene, ζ-carotene, and neurosporene. Phytoene accumulation does not visibly alter the color of E. coli. Thus, we could not observe a color change in the culture upon induction of LeCCD1A or LeCCD1B. However, a color change was observed in E. coli overexpressing the CCD1 proteins in ζ-carotene-accumulating strains. Taken together, the in vitro and in vivo results indicate that the CCD1 enzymes have broad substrate specificity, recognizing both acyclic and cyclic carotenoids.

A significant decrease (approximately 90%) in LeCCD1 expression was accompanied by only 40–60% reduction in β-ionone or geranylacetone in vivo. The lack of a direct correlation between RNA accumulation and product is not in itself surprising. It may, however, indicate the existence of a redundant activity. That there is another CCD enzyme capable of 9,10 cleavage is supported by the results of Booker et al. (2004), who identified a chloroplast-localized carotenoid cleavage dioxygenase (AtCCD7/AtMAX3) from Arabidopsis. AtCCD7 catalyzes the oxidative cleavage of β-carotene at the 9,10 double bond resulting in formation of β-ionone and β-apo-10’-carotenal, a C27 aldehyde. Furthermore, an AtCCD7 ortholog from mouse, β-β-carotene-9,10-dioxygenase, has also been reported to cleave both β-carotene and lycopene at the 9,10 double bond (Kiefer et al., 2001). The presence of a CCD7 homolog in tomato (A.J. Simkin and H.J. Klee, unpublished data) raises the possibility of another enzyme responsible for generating at least a portion of the β-ionone and geranyl acetone in fruits. As there is significant sequence divergence between the CCD1 and CCD7 genes (38% identity), we would not expect any antisense-suppression of LeCCD7 in these plants.

We also cannot exclude photooxidation as an additional mechanism for the formation of β-ionone, pseudoionone, or geranylacetone. The formation of β-ionone from β-carotene by free radical-mediated cleavage of the 9-10 bond has previously been demonstrated in vitro (Wache et al., 2003). As much as 1 mg of carotenoids day⁻¹ g⁻¹ DW are oxidized in pepper leaves (Simkin et al., 2003). During tomato fruit ripening, the concentration of carotenoids increases between 10- and 14-fold, due mainly to the accumulation of lycopene (Fraser et al., 1994). Given the overall quantity of carotenoids that accumulate during fruit ripening (Fraser et al., 1994), the rates of both β-ionone and geranylacetone emission are very low. One reason for the low rate of synthesis of these apocarotenoids may be lower availability of the substrate to a non-plastid targeted enzyme. In vitro import assays have shown that LeCCD1A and LeCCD1B are located in the cytosol and/or attached to the outer envelope of pea chloroplast. We have also verified that result with LeCCD1B:GFP fusions in tobacco protoplasts (I. Mila, M. Bouzayen and H.J. Klee, unpublished data). This is consistent with previously reported results for the Arabidopsis homolog AtCCD1 (Tan et al., 2003) and the C. sativus homolog CsCCD (Bouvier et al., 2003b). LeCCD1 access to substrates may be limited to carotenoids located in the outer envelope. However, the outer envelope of chloroplasts contains significant quantities of carotenoids (Block et al., 1983; Markwell et al., 1992).

Possible biological functions of LeCCD1 enzymes

Although we did observe significant reductions in the rates of emission of β-ionone and geranylacetone in cut tomato fruits, we did not observe significant changes in the carotenoid content of these fruits. In pepper chloroplasts cleavage is a minor process in carotenoid turnover (Simkin et al., 2003). Given that LeCCD1A and LeCCD1B are not plastid-localized, it is not unexpected that plants with greatly reduced LeCCD1 expression showed insignificant alterations in carotenoid content. Thus, it would appear that these enzymes do not have a significant role in carotenoid turnover.

Real-time RT-PCR results indicate that both LeCCD1A and LeCCD1B are expressed in fruits. It is likely that LeCCD1B is the dominant contributor to the synthesis of β-ionone, geranylacetone, and pseudoionone in fruit due to its abundance during fruit development. Peak levels of β-ionone and geranylacetone emissions at ripe fruit were calculated to be 1.25 and 40 pg g⁻¹ fw h⁻¹, respectively. Although β-ionone and geranylacetone are found in low concentrations when compared with other more abundant volatiles such as cis-3-hexenal and hexenal, which have been detected at levels 10 000-fold higher, β-ionone and geranylacetone have odor thresholds of 0.007 nl l⁻¹ and 60 nl l⁻¹, respectively (Baldwin et al., 2000). This odor threshold is significantly lower than that observed for many of the other more abundant volatiles. Thus, these carotenoid-derived volatiles have the potential to greatly impact aroma and...
flavor at low concentrations. β-ionone is considered to be the second most important volatile contributor to tomato fruit flavor (Baldwin et al., 2000).

In addition to fruits, there is also significant expression of LeCCD1A and LeCCD1B in roots and leaves. It is not clear what role expression of these genes would have in vegetative tissues. The 9,10 symmetric cleavage of diverse carotenoids by CCD1 would result in the formation of a variety of C13 cyclohexene apocarotenoids, depending on the substrate, and a C14 dialdehyde, corresponding to the central portion of the original carotenoid precursor (Schwartz et al., 2001). This C14 dialdehyde is thought to be the precursor of mycorradicin, a yellow pigment that accumulates in the roots of plants infected with arbuscular mycorrhizal fungi (Walter et al., 2000). Several C13 cyclohexene derivatives have also been identified in the same root tissue (Maier et al., 1995, 1997, 2000; Walter et al., 2000).

Application of blumenin, a C13 carotenoid-derived product that accumulates in roots (Maier et al., 1995; Walter et al., 2000), strongly inhibits early fungal colonization and arbuscule formation, implying that cyclohexenone derivatives might act in the plant to control fungal spread (Fester et al., 1999). β-ionone inhibits the growth of multiple pathogenic fungi, including Peronospora tabacina (Schiltz, 1974), Colletotrichum musae (Utama et al., 2002), Fusarium solani, Botrytis cinerea, and Verticillium dahlieae (Mikhlin et al., 1983). Thus, it is possible that expression of LeCCD1A and LeCCD1B in vegetative tissues and fruit may have a role in the formation of multiple antimicrobial compounds. Furthermore, the CCD1 C13 product of zeaxanthin or lutein cleavage, 3-hydroxy-β-ionone, accumulates in etiolated bean seedlings on exposure to light. This compound may have a function in the light-induced inhibition of hypocotyl elongation (Kato-Noguchi, 1992; Kato-Noguchi et al., 1993).

Conclusions

We have demonstrated that LeCCD1A and LeCCD1B cleave multiple linear and cyclic carotenoids resulting in the formation of a C14 dialdehyde and a variety of C13 products, depending on the substrate. These enzymes contribute to the formation of β-ionone and geranlylactone, important constituents of tomato flavor and aroma in vivo. These results point to the possibility of manipulating the flavor profiles of tomato fruits through genetic manipulation of LeCCD1 gene products. Many of these volatile compounds, due to their low odor threshold, require only small amounts to affect a change in flavor. The availability of plants altered in CCD1 expression places us in a position to assess the function of these products in vivo as well as their specific contributions to tomato flavor.

Experimental procedures

Plant material and treatment

Tomato (Lycopersicon esculentum, cv. M82) plants were grown under greenhouse conditions in commercial potting medium (Metromix 500; Scotts, Marysville, OH, USA) in 27-cm, 12-L pots, supplemented with time release fertilizer (Osmocote 14-14-14; Scotts). For developmental studies, fruits were divided into seven developmental stages from 1 to 5 cm in diameter. The fruits were green through stages 1–4, breaking (first sign of color) at stage 5 and turning orange and red at stages 6 and 7, respectively. Leaf tissue was taken from newly developing leaves at the growing tip or the plant. Flowers were collected when fully open and roots collected from plants shortly after the development of the first flower.

Transgenic tomato plants were produced using Agrobacterium-mediated transformation (McCormick et al., 1986). A partial-length cDNA of LeCCD1B from the 3’ end (positions 940–1638) was cut from pBluescript SK+ with BamHI and KpnI and introduced in the antisense orientation under the control of the FMV 35S promoter (Richins et al., 1987), and followed by the nos 3’ terminator to make pHK-LeCDD1. The vector is a binary transformation vector which was introduced into Agrobacterium strain ABI. Transformed plant tissue was selected for resistance to kanamycin. Introduction and inheritance of the transgenes were confirmed by PCR using primers specific for the selectable marker gene (NPTII).

Co-expression of LeCCD1 in carotenoid accumulating strains of E. coli

An LeCCD1A cDNA containing the complete open-reading frame of 1638 bp was recovered in a continuous piece by RT-PCR from leaf tissue. Oligonucleotides for amplification of the cDNA were based on the sequence of an apparently full-length contig sequence in the TIGR database (TC124799). The fragment was amplified with the following oligonucleotides: 5’ oligo – CACCGAAAAAGTT- GTTATCAAAAAAAAATG; 3’ oligo – GCCAGTAGCTATTGCTTCA. This PCR product was then cloned directly into the pENTRD vector (Invitrogen). LeCCD1B also has an open-reading frame of 1638 bp and was recovered in three independent overlapping pieces. An initial 3’ fragment of 631 nt was isolated from a partial EST clone (cLEF40015). The 5’ fragments were amplified by 5’-RACE. The full sequence was obtained by simultaneously ligating the three pieces at the Haelli site (position 216) and TaaI site (position 990) to give a full-length open-reading frame of 1638 bp in pENTRD. The LeCCD1A and LeCCD1B full-length cDNA sequences were transferred into either the pDEST14 or pDEST15-GST vectors (Invitrogen) by recombination. Plasmids containing the carotenoid biosynthetic genes (Cunningham and Gantt, 2001; Cunningham et al., 1994, 1996; Sun et al., 1996) were co-transformed into E. coli strain BL21-AI with the pDEST14-LeCCD1A or pDEST14-LeCCD1B constructs described above. Controls consisted of carotenoid-producing strains without the LeCCD1 genes subjected to identical induction treatments. An overnight culture of 3 ml was used to inoculate 50 ml of LB medium containing the appropriate antibiotics and 0.2% glucose. The cultures were grown at 28°C until an absorbance at 600 nm of 0.5 was reached. Expression of the protein was induced by addition of 0.2% arabinose and the cultures were grown at 28°C overnight. Two milliliters of the E. coli culture was centrifuged, and the cell pellet was photographed. The remaining E. coli culture was centrifuged, and the cell pellet was resuspended in an equal volume of formaldehyde. An equal volume of methanol was then added, followed by two volumes of ethyl acetate. The phases were
separated by the addition of water, and the ethyl acetate phase was retained for HPLC analysis. Following centrifugation, the medium from the cultures was partitioned twice with an equal volume of ethyl acetate. The ethyl acetate was evaporated under vacuum, and the extracts were saponified prior to HPLC analysis. The HPLC fractions were dried, resuspended in the appropriate solvent, and used for UV-visible or fluorescence spectroscopy. A rosafuene standard was prepared by the reduction of the C14 dialdehyde with NaBH₄.

**Protein expression and enzyme assays in vitro**

A 5-ml culture of the pDEST15-LeCCD1A or pDEST15-LeCCD1B was used to inoculate a 100-ml culture in 2X-YT medium (per liter: 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl). Cultures were grown at 37°C until an A600 of 0.7 was reached. Expression of the protein was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside, and the cultures were grown at 28°C for an additional 3-5 h. The E. coli cells were harvested by centrifugation, resuspended in Tris-buffered saline, and lysed in a French press. The recombinant protein was purified with glutathione S-transferase-agarose (Sigma, St Louis, MO, USA) and then eluted from the column with reduced glutathione, which was subsequently removed by chromatography on Sephadex G-25. The carotenoid substrates were extracted and purified as previously described (Rock and Zeevaart, 1991). Assays contained 0.1% Triton X-100, 0.5 mM FeSO₄, 5 mM ascorbate, and the appropriate carotenoid substrate in 100 mM Bis–Tris pH 7.0. The assay products were partitioned into ethidium bromide. TaqMan one-step real-time RT-PCR was carried out using 250 ng of total RNA samples in each experiment by formaldehyde agarose gel electrophoresis and visual inspection of rRNA bands upon ethidium bromide staining. TaqMan one-step real-time RT-PCR was carried out using primer express software (Applied Biosystems; see Table 1).

**Extraction of total RNA and real-time quantitative RT-PCR**

Total RNA was isolated from tissues using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). RNA samples were treated with RNase-free DNase (Qiagen) and purified using the Qiagen mini-column. Samples were checked for DNA contamination by TaqMan real-time RT-PCR in a reverse-minus transcription reaction. Concentration and purity of total plant RNA was determined by spectrophotometric analysis. The quantification was verified for all RNA samples in each experiment by formaldehyde agarose gel electrophoresis and visual inspection of rRNA bands upon ethidium bromide staining. TaqMan one-step real-time RT-PCR was carried out as recommended by the manufacturer (Applied Biosystems). All reactions contained 1× TaqMan buffer (Applied Biosystems) and 5 mM MgCl₂, 200 μM each of dATP, dCTP, dGTP, and 400 μM dUTP, 0.625 units of AmpliTaq Gold polymerase, and 0.25 units of MultiScribe reverse transcriptase and RNase inhibitor in a 25 μl volume. Reverse transcription was carried out using 250 ng of total RNA, 200 nM of each gene-specific primers, forward and reverse, and 100 nM TaqMan probe. Primers and probes were designed using primer express software (Applied Biosystems; see Table 1). Cross-reactivity of each probe to the other gene product was determined to be <10⁻⁶. Reaction mixture was incubated for 30 min at 48°C for reverse transcription, 10 min at 95°C followed by 40 amplification cycles of 15 sec at 95°C/1 min at 60°C. Samples were quantified in the GeneAmp 7500 Sequence Detection System (Perkin-Elmer). Absolute mRNA levels were quantified against a standard curve of tritium quantified LeCCD1A and LeCCD1B in vitro transcribed sense strand RNAs.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers and probes used in TaqMan real-time quantitative RT-PCR assay. Primers and probes were designed using PRIMER EXPRESS software (Applied Biosystems)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LeCCD1A</td>
<td><strong>Forward</strong> TTGGATACCTGCGCCGCTGTG <strong>Reverse</strong> CATATAGCTATTGACGAAATTC <strong>Probe</strong> ACCCAGACTGACATGGTCAATGGACG</td>
</tr>
<tr>
<td>LeCCD1B</td>
<td><strong>Forward</strong> AGAAATCCGACAGTCTTACGCGATT <strong>Reverse</strong> CCTCTCTCATACATCAGCTTTG <strong>Probe</strong> AGGAACTGAAAAGAAGAACAGGGC</td>
</tr>
</tbody>
</table>

*All probes were labeled at the 5’ with fluorescent reporter dye 6-carboxyfluorescein and at the 3’ with black hole quencher-1 (BHQ-1) from Integrated DNA Technologies (Coralville, IA, USA) (Bernacchi and Mély, 2001).

In vitro transcription, translation, and import assay

All constructs for in vitro transcription were made under the SP6 promoter in pSP64 poly(A) vector (Promega, Madison, WI, USA). The LeCCD1A full-length cDNA sequence was recovered by PCR using primers AA-PstI-ATGGAGAAGAAAAGATGA and CG-BamHI-TCACAGTGGTGCATTCTGAATTGTGC. For LeCCD1B, the cDNA sequence was recovered using primers XbaI-ATGGGATGAAAGAAGA and CG-BamHI-TCACATTTTGCCGGCTCTGAATTGGC. The PCRs were digested with PstI (LeCCD1A) or XbaI (LeCCD1B) and ligated into the pSP6 vector (Promega). The DNA sequences encoding ribulose 1,5-bisphosphate carboxylase small subunit precursor from *P. sativum* L., transcribed from plasmids containing the SP6 promoter (Anderson and Smith, 1986), was used as a positive control.

Intact chloroplasts were isolated at 4°C from 9 to 10 day-old pea (*P. sativum* cv. Laxton’s progress 9) seedlings (Cline, 1986) in isolation buffer (1 mM sodium pyrophosphate (Na₄P₂O₇), 50 mM HepES, 0.33 mM sorbitol, 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 1 mM β-mercaptoethanol, pH 6.8). Chloroplasts were resuspended in import buffer and kept on ice until use. In vitro transcription, translation, and import were carried out using the TNT Coupled Reticulocyte Lysate Systems (Promega) as described previously (Boeker et al., 2004).

Isoprenoid analysis and detection by HPLC-PDA

The method used to analyze and quantify carotenoids is detailed in Fraser et al. (2000). Typically, tomato tissues were freeze-dried and ground into a powder, 10–50 mg aliquots were extracted using methanol/chloroform (1:3, V/V) and partitioned against 50 mM Tris–HCl pH 7.0 (2 vol.). The aqueous phase was re-extracted twice. HPLC separations were performed on a C30 reverse-phase column (250 x 4.6 mm) (Waters, Milford, MA, USA). The mobile phases used were methanol (A), water/methanol (20/80 V:V) containing 0.2% ammonium acetate (B) and tert-methyl butyl ether (C). The gradient used was 95% A/5% B isocratically for 10 min, a step to 80% A/5% B and 15% C at 10 min, followed by a linear gradient to 30% A/5% B/65% C by 30 min (Fraser and Sandmann, 1992; Fraser et al., 2000).

Volatle analysis

Diced fruit were placed into glass vessels and assayed under normal light conditions. Experiments utilized glass cylinders (17 mm
Carotenoid cleavage dioxygenases of Lycopersicon esculentum

i.d. × 61 cm long, 127 ml volume) and collection of volatiles followed Turlings et al. (1991). Briefly, clean humidified air was passed through the vessels (550 ml min⁻¹) and volatiles were trapped on 30 mg Super Q (80/100 mesh; Alltech, Deerfield, IL, USA). The Super Q traps were eluted with 150 μl dichloromethane and 400 ng nonyl acetate (in 5 μl dichloromethane) was added as an internal standard. Separation of volatiles was performed on an Agilent 6890N gas chromatograph according to Schmelz et al. (2001). Quantification was carried out using a standard curve of β-ionone or geranylacetone (Sigma).

Acknowledgements

We would like to thank Gina Fonfara for helping with volatile analysis and Carole Dabney-Smith for the SP64-LHCP and SP64-SRSU plasmids and invaluable assistance in the chloroplast uptake experiments. We would also like to thank Dr F. Cunningham for the plasmids used in carotenoid accumulating strains of E. coli and Yec’han Laizet for sequence alignments. We would also like to gratefully acknowledge Eran Pichersky for his careful and thoughtful advice on the manuscript. This work was funded in part by National Science Foundation grant IBN0115004 to HK. This is publication R-10462 of the Florida Agricultural Experiment Station.

Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2263/TPJJ2263sm.htm

Table S1 Alignment of CCD1 proteins. The predicted amino acid sequences of the tomato carotenoid cleavage dioxygenases CdC1A (AY576001) and CdC1B (AY576002) aligned with related proteins from Arabidopsis thaliana, Phaseolus vulgaris and capsicum annum.

References


