Carotene Hydroxylase Activity Determines the Levels of Both α-Carotene and Total Carotenoids in Orange Carrots

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The typically intense carotenoid accumulation in cultivated orange-rooted carrots (Daucus carota) is determined by a high protein abundance of the rate-limiting enzyme for carotenoid biosynthesis, phytoene synthase (PSY), as compared with white-rooted cultivars. However, in contrast to other carotenoid accumulating systems, orange carrots are characterized by unusually high levels of α-carotene in addition to β-carotene. We found similarly increased α-carotene levels in leaves of orange carrots compared with white-rooted cultivars. This has also been observed in the Arabidopsis thaliana lut5 mutant carrying a defective carotene hydroxylase CYP97A3 gene. In fact, overexpression of CYP97A3 in orange carrots restored leaf carotenoid patterns almost to those found in white-rooted cultivars and strongly reduced α-carotene levels in the roots. Unexpectedly, this was accompanied by a 30 to 50% reduction in total root carotenoids and correlated with reduced PSY protein levels while PSY expression was unchanged. This suggests a negative feedback emerging from carotenoid metabolites determining PSY protein levels and, thus, total carotenoid flux. Furthermore, we identified a deficient CYP97A3 allele containing a frame-shift insertion in orange carrots. Association mapping analysis using a large carrot population revealed a significant association of this polymorphism with both α-carotene content and the α/β-carotene ratio and explained a large proportion of the observed variation in carrots.

INTRODUCTION

Carrot (Daucus carota) roots exhibit a panoply of colors ranging from yellow to orange and red (Surles et al., 2004; Arscott and Tanumihardjo, 2010). This color spectrum is mostly determined by the abundance of carotenoids, with the exception of purple carrots, which also accumulate anthocyanins (Simon et al., 2008; Montilla et al., 2011). The earliest cultivated carrots were yellow or purple and emerged in the Afghanistan region before the 900s, while orange and white cultivars were bred in the 16th century (Bangal, 1957; Soufflet-Freslon et al., 2013). Despite significant progress in the identification of genetic markers associated with root color in recent years, knowledge of the molecular basis of divergent color phenotypes is sparse (Santos and Simon, 2006; Just et al., 2009; Fuentes et al., 2012; Rodriguez-Concepcion and Stange, 2013).

In carrot roots, carotenoids accumulate as crystals within chloroplasts (Maass et al., 2009; Kim et al., 2010; Wang et al., 2013). In leaf chloroplasts, these pigments are mostly protein-bound, forming essential constituents of light-harvesting complexes and photosynthetic reaction centers. Furthermore, carotenoids represent ultimate precursors for at least two classes of plant hormones, abscisic acid (ABA) and the strigolactones (Walter and Strack, 2011; Alder et al., 2012).

The first carotenoid-specific reaction of plastid prenyl lipid biosynthesis is catalyzed by the enzyme phytoene synthase (PSY; Figure 1) and yields the C40 hydrocarbon phytoene (for a review on carotenoid biosynthesis, see Cazzonelli, 2011). Following desaturation and isomerization reactions, which are catalyzed by four enzymes in plants (phytoene desaturase, ζ-carotene desaturase, ζ-carotene isomerase, and carotenoid isomerase), the red-colored all-trans-lycopene is formed (Isaacsion et al., 2002; Park et al., 2002; Li et al., 2007). Two different cyclases introduce ε-ionone (ε-cyclase, LCYε) and/or β-ionone rings (β-cyclase, LCYb), yielding α-carotene (ε/β-carotene) or β-carotene (β/β-carotene), respectively.

The synthesis of the oxygen-containing xanthophylls from either α- or β-carotene requires ring-specific hydroxylation reactions. In Arabidopsis thaliana, these reactions are catalyzed by a set of four enzymes (Kim et al., 2009). Two non-heme di-iron enzymes (β-carotene hydroxylase 1 [BCH1] and BCH2) are primarily responsible for β-ring hydroxylation of β-carotene and produce zeaxanthin, while two heme-containing cytochrome P450 enzymes (CYP97A3 and CYP97C1) preferentially hydroxylate the ε- and β-ionone rings of α-carotene, yielding lutein. Zeaxanthin is further epoxidized by the enzyme zeaxanthin epoxidase, leading to antheraxanthin, violaxanthin, and neoxanthin, a reversible reaction representing the xanthophyll cycle (Nyogoi et al., 1998). The analysis of leaf carotenoid patterns from hydroxylase-deficient Arabidopsis mutants revealed partially overlapping substrate specificities of the hydroxylases involved and reflect the requirement for the complete set of hydroxylases to generate a balanced pigment composition (Kim et al., 2009).

High carotenoid levels in non-green plant tissues have frequently been found to be dependent on PSY expression, indicating this enzyme to be rate-limiting in carotenogenesis. The overexpression of PSY, e.g., in tomato fruits (Solanum lycopersicum), cassava
roots (Manihot esculenta), canola seeds (Brassica napus), and rice endosperm (Oryza sativa), effectively increased the flux through the biosynthetic pathway (Shewmaker et al., 1999; Ye et al., 2000; Fraser et al., 2007; Welsch et al., 2010). Fluxes can be driven at levels where even δ-carotene crystals form. This was observed in transgenic white-rooted carrots overexpressing the bacterial PSY (crtB; Maass et al., 2009). Accordingly, high PSY protein levels can be revealed by immunoblotting in orange-rooted cultivars while being undetectable in white-rooted cultivars. However, these large differences are not equivalently reflected in PSY mRNA levels, suggesting the involvement of additional regulatory mechanisms. Identification of the remaining genes involved in carotenoid biosynthesis permitted analysis of their expression during root development of various color cultivars (Clotault et al., 2008). However, the results obtained can only partially explain the different carotenoid patterns and fall to account for the wide range of carotenoid levels found.

Compared with other carotenoid accumulating tissues, one remarkable feature of orange carrots is the high abundance of α-carotene, in addition to β-carotene. We found that unusually high α-carotene levels are also present in leaves of orange-rooted carrots. This is a distinctive characteristic of plants with a deficiency in one cytochrome P450-type carotene hydroxylase, as concluded from investigations of Arabidopsis mutants (Kim and Dellapenna, 2006). In this work, we took advantage of this knowledge and investigated whether the high α-carotene phenotype in carrots is attributed to such a defect by overexpressing the corresponding Arabidopsis carotene hydroxylase gene in orange-rooted carrots. Our data show that this assumption is correct inasmuch as the carotenoid pattern is concerned and identified a feedback mechanism on total carotenoid content that might be involved in carotenoid differences observed in various carrot varieties.

RESULTS

α-Carotene Levels in Carrot Cultivars

Most cultivated orange carrots exhibit remarkably high α-carotene levels, between 15 and 30% of the total carotenoid content, and an accordingly high α/β-carotene ratio of 0.2 up to 0.5 (Surles et al., 2004; Clotault et al., 2008; Simon et al., 2008; Arscott and Tanumihardjo, 2010). For instance, tomato fruits accumulate ~4% α-carotene with an α/β-carotene ratio of 0.02 (Stigliani et al., 2011), and Arabidopsis leaves contain <1% α-carotene with an α/β-carotene ratio of ~0.03 (Kim et al., 2009). Furthermore, root-specific overexpression of PSY in a white-rooted carrot background (cultivar Queen Anne’s Lace [QAL]) increases the total carotenoid amounts, but this is almost exclusively due to elevated β-carotene while α-carotene remains at low levels. Similarly, PSY overexpression in Arabidopsis roots results in significant carotenoid accumulations with only 3% α-carotene and low α/β-carotene ratios (Maass et al., 2009).

We compared the carotenoid profiles in leaves from two orange-rooted cultivars (Chantenay Red Cored [CRC] and Nantaise [NANI]) with two white-rooted cultivars (QAL and Küttinger [KUT]), which accumulate high and low PSY protein levels, respectively (Maass et al., 2009). Table 1 shows that irrespective of root color and PSY abundance, total leaf carotenoid levels and the carotenoid/chlorophyll ratios were very similar and so were the levels of the major xanthophyll lutein, an α-carotene derivative. The difference to be noted is that the proportion of α-carotene is strongly elevated, up to 10% of the total carotenoids, in leaves of orange-rooted cultivars, compared with those of white-rooted cultivars where only ~1% α-carotene was found. Based on previous results where α-carotene levels were unchanged upon overexpression of PSY (lines At12 and At22 in Table 1; Maass et al., 2009), we conclude that high levels of α-carotene in leaves, and supposedly also in roots of orange carrots, involve additional mechanisms that are present in orange but absent from white-rooted cultivars.

Alteration in ε-ring hydroxylation pattern affecting α-carotene levels are known from Arabidopsis mutants with reduced carotene hydroxylase capacity (Kim et al., 2009). Among the cytochrome
The similarity in the leaf carotenoid patterns between Rescue the Leaf Phenotype detected in CRC and NAN, while being absent in QAL and KUT.

CYP97A3 activity in orange cultivars. Aiming at rescuing the low DJ3Spro:AtCYP97A3 and Causes a Reduction in Total Carotenoid Levels grown in soil and their roots harvested after 8, 12, and 16 weeks.

The increase of \( \alpha \)-carotene in \( lut5 \) is compensated for by an almost equivalent re-

As with the leaves, \( \alpha \)-carotene was found strongly reduced in these roots throughout all stages, comprising only \(-2\% of total carotenoids compared with 20\% in untransformed CRC control plants (Figure 3B; Supplemental Figure 3). Accordingly, the \( \alpha/\beta \)-carotene ratio decreased from 0.5 in nontransformed CRC to 0.1 and 0.04 in average in lines Dc#1 and Dc#25, respectively; this decrease was \(-3\)-fold during root growth (Figure 3E). Young roots of transgenic lines also accumulated CYP97A3-catalyzed monohydroxylated intermediates, such as zeinoxanthin and \( \beta \)-cryptoxanthin, which were absent in the controls (Supplemental Table 2). These intermediates disappeared in older transgenic roots (12 and 16 weeks), suggesting a limited activity of other carotene hydroxylases, e.g., the \( \epsilon \)-ring specific CYP97C1, during early root development. Surprisingly, the decrease in \( \alpha \)-carotene from \(-300 \mu g \) in untransformed CRC to 10 \( \mu g \) in line Dc#25 did not entail an equivalent increase in CYP97A3-derived xanthophylls. These remained at only small total amounts in both young roots (100 \( \mu g \) in Dc#25 versus 71 \( \mu g \) in CRC) as well as older roots (150 \( \mu g \) in Dc#25 versus 100 \( \mu g \) in CRC).

The similarity in the leaf carotenoid patterns between Arabidopsis \( lut5 \) and orange-rooted carrots suggests the presence of reduced CYP97A3 activity in orange cultivars. Aiming at rescuing the low \( \alpha \)-carotene "chemyotype," we transformed the orange carrot cultivar CRC with a construct harboring the CYP97A3 cDNA from Arabidopsis (At-CYP97A3; Kim and DellaPenna, 2006) under control of the DJ3S promoter from yams. This promoter is highly active in Arabidopsis CYP97A3 lines, Arabidopsis wild-type and \( lut5 \) leaves, confirmed the presence of the mature At-CYP97A3 protein and mirrored overall transgene expression differences between these lines (Figure 3C).

Table 1. Carotenoids in Arabidopsis and Carrot Leaves

<table>
<thead>
<tr>
<th>Line</th>
<th>Tot. Car</th>
<th>Lutein</th>
<th>( \alpha )-Crypt</th>
<th>( \alpha )-Carotene</th>
<th>( \beta )-Carotene</th>
<th>VAZN</th>
<th>chla/b</th>
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<tbody>
<tr>
<td>QAL</td>
<td>431.4 ± 2</td>
<td>206.6 ± 0 (47.9)</td>
<td>ND</td>
<td>2.6 ± 0 (0.6)</td>
<td>84.0 ± 1 (19.5)</td>
<td>138.2 ± 1 (32.0)</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>KUT</td>
<td>458.1 ± 36</td>
<td>213.4 ± 3 (46.8)</td>
<td>ND</td>
<td>3.4 ± 1 (0.7)</td>
<td>81.5 ± 17 (17.6)</td>
<td>159.8 ± 15 (34.8)</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>CRC</td>
<td>430.0 ± 8</td>
<td>213.4 ± 3 (48.7)</td>
<td>1.4 ± 0.3 (0.3)</td>
<td>26.1 ± 1 (6.0)</td>
<td>64.9 ± 4 (14.8)</td>
<td>132.3 ± 0 (30.2)</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>NAN</td>
<td>458.3 ± 2</td>
<td>222.8 ± 5 (48.6)</td>
<td>6.9 ± 1.1 (1.5)</td>
<td>45.7 ± 5 (10.0)</td>
<td>59.6 ± 8 (13.0)</td>
<td>123.2 ± 5 (26.9)</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>At-Wt</td>
<td>521.7 ± 24</td>
<td>238.5 ± 30 (45.0)</td>
<td>ND</td>
<td>3.9 ± 1 (0.7)</td>
<td>114.6 ± 19 (22.9)</td>
<td>169.5 ± 44 (32.3)</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>At12</td>
<td>486.2 ± 31</td>
<td>227.0 ± 14 (46.7)</td>
<td>ND</td>
<td>3.1 ± 1 (0.6)</td>
<td>105.8 ± 10 (21.7)</td>
<td>150.8 ± 9 (30.9)</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>At22</td>
<td>534.0 ± 2</td>
<td>248.6 ± 6 (48.6)</td>
<td>ND</td>
<td>2.4 ± 2 (0.4)</td>
<td>107.6 ± 15 (20.2)</td>
<td>175.4 ± 24 (32.9)</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>( lut5-1 )</td>
<td>503.8 ± 18</td>
<td>248.1 ± 6 (49.3)</td>
<td>8.9 ± 0.9 (1.8)</td>
<td>78.9 ± 4 (15.7)</td>
<td>45.5 ± 7 (9.0)</td>
<td>122.4 ± 8 (24.3)</td>
<td>3.1 ± 0.1</td>
</tr>
</tbody>
</table>

Carotenoids were quantify by HPLC from leaves of white-rooted (QAL and KUT) and orange-rooted carrot cultivars (CRC and NAN). For comparison, leaf carotenoids are shown from two CaMV35Spro:At-PSY Arabidopsis lines (At12 and At22), the corresponding wild type (At-Wt), and from the \( lut5-1 \) mutant carrying a T-DNA integration within CYP97A3. Carotenoids are expressed in mmol pigment mol\(^{-1}\) chlorophyll. The relative percentage of each carotenoid is given in parentheses. Tot. Car, total carotenoids; \( \alpha \)-Cryp, \( \alpha \)-cryptoxanthin; VAZN, sum of violaxanthin, antheraxanthin, zeaxanthin, and neoxanthin; chla/b, ratio between chlorophyll \( a \) and \( b \); ND, not detectable. Data represent the mean ± so of four (carrot) and three (Arabidopsis) replicates, respectively.

P450 (CYP97C1 and CYP97A3) and non-heme hydroxylases (BCH1 and BCH2) involved, only the CYP97A3 mutant showed a high proportion of \( \alpha \)-carotene (between 15 and 20\%), exhibiting the \( lut5 \) phenotype (Kim and DellaPenna, 2006). Moreover, the \( \beta \)-carotene ratio decreased from 0.5 in nontransformed CRC control roots of the same age (Figure 3A). Furthermore, immunoblot analysis performed with roots from two transgenic DJ3Spro:AtCYP97A3 lines, Arabidopsis wild-type and \( lut5 \) leaves, confirmed the presence of the mature At-CYP97A3 protein and mirrored overall transgene expression differences between these lines (Figure 3C).

Expression of Arabidopsis CYP97A3 in Orange Carrots Rescues the Leaf Phenotype

Root-Specific Overexpression of Arabidopsis CYP97A3 in Orange Carrots Strongly Reduces \( \alpha \)-Carotene Amounts and Causes a Reduction in Total Carotenoid Levels

\( DJ3Spro:AtCYP97A3 \) CRC lines and wild-type CRC carrots were grown in soil and their roots harvested after 8, 12, and 16 weeks.

Growth of transgenic lines was indistinguishable from non-transformed CRC and roots developed similar to the wild type (Supplemental Table 2). At-CYP97A3 mRNA was strongly expressed throughout all selected stages (Figure 3A). Furthermore, immunoblot analysis performed with roots from two transgenic DJ3Spro:AtCYP97A3 lines, Arabidopsis wild-type and \( lut5 \) leaves, confirmed the presence of the mature At-CYP97A3 protein and mirrored overall transgene expression differences between these lines (Figure 3C).

As with the leaves, \( \alpha \)-carotene was found strongly reduced in these roots throughout all stages, comprising only \(-2\% of total carotenoids compared with 20\% in untransformed CRC control plants (Figure 3B; Supplemental Figure 3). Accordingly, the \( \alpha/\beta \)-carotene ratio decreased from 0.5 in nontransformed CRC to 0.1 and 0.04 in average in lines Dc#1 and Dc#25, respectively; this decrease was \(-3\)-fold during root growth (Figure 3E). Young roots of transgenic lines also accumulated CYP97A3-catalyzed monohydroxylated intermediates, such as zeinoxanthin and \( \beta \)-cryptoxanthin, which were absent in the controls (Supplemental Table 2). These intermediates disappeared in older transgenic roots (12 and 16 weeks), suggesting a limited activity of other carotene hydroxylases, e.g., the \( \epsilon \)-ring specific CYP97C1, during early root development. Surprisingly, the decrease in \( \alpha \)-carotene from \(-300 \mu g \) in untransformed CRC to 10 \( \mu g \) in line Dc#25 did not entail an equivalent increase in CYP97A3-derived xanthophylls. These remained at only small total amounts in both young roots (100 \( \mu g \) in Dc#25 versus 71 \( \mu g \) in CRC) as well as older roots (150 \( \mu g \) in Dc#25 versus 100 \( \mu g \) in CRC).

Moreover, the conceivable decrease in \( \alpha \)-carotene was accompanied by an unexpected 30 to 50\% reduction in total carotenoid levels in transgenic DJ3Spro:AtCYP97A3 relative to untransformed control roots of the same age (Figures 3B and 3D). This decrease affected not only \( \beta \)-carotene, but all other carotenoids, including desaturation intermediates such as phytoene, phytofluene, and \( \zeta \)-carotene. For instance, \( \beta \)-carotene was reduced by 77\%, while noncolored carotenones were reduced by 32\% in 8-week-old roots from line Dc#25 compared with the control. Expression of the putative carrot homolog of the ABA-responsive gene 9-cis-epoxycarotenoid dioxygenase 3 (NCED3) was largely unchanged in the transgenic lines (Supplemental Figure 4; Auldridge et al., 2006; Just et al., 2007). This suggests that the altered carotenoid levels did not entail changes in ABA synthesis.
The Plant Cell

Figure 2. α-Carotene and α-/β-Carotene Ratio in Leaves from DJ3Spro:AtCYP97A3 Carrots.

Orange-rooted carrots (CRC) were transformed with At-CYP97A3 under control of the yam DJ3S promoter (DJ3Spro). Both leaf α-carotene levels (A) as well as α-/β-carotene ratios (B) are strongly reduced in DJ3Spro:AtCYP97A3 lines (Dc#1, Dc#25, and Dc#7) and approached levels determined in leaves of white-rooted carrots (QAL). α-Carotene content is given in mmol mol⁻¹ chlorophyll. Data are mean of four (controls) and three (transgenic lines) biological replicates ± sd, respectively.

Since PSY has frequently been reported as the rate-limiting step in non-green plant tissues (Fraser et al., 2002; Paine et al., 2005; Rodríguez-Villalón et al., 2009; Welsch et al., 2010), reduced phytoene and total carotenoid levels may suggest an attenuated phytoene synthesis capacity as a consequence of At-CYP97A3 overexpression. However, transcript levels of both carrot PSY genes were quite similar in all lines throughout all stages compared with nontransformed CRC carrots (Figure 4A). We therefore investigated PSY protein levels in roots from DJ3Spro:AtCYP97A3 lines and nontransformed CRC carrots. Protein gel blot analysis revealed that, in fact, PSY protein amounts were very strongly reduced, e.g., by ~50% in line Dc#25 (Figures 4B and 4C). Levels of a putative PSY degradation product did not increase, which suggests that PSY translation rather than its turnover rate is affected. Furthermore, Arabidopsis wild-type and lut5 roots accumulate barely detectable PSY protein levels, which appear somewhat higher in the mutant (Supplemental Figure 5). This might indicate that the observed dependence of PSY protein levels on hydroxylation capacity is not only a distinct feature of carrots. In conclusion, the altered carotene hydroxylation upon At-CYP97A3 overexpression in orange carrots negatively affected PSY protein levels, explaining the reduced total carotenoid accumulation observed.

Cloning of CYP97A3 from White Carrots

High α-carotene levels in CRC wild type and their strong reduction upon Arabidopsis CYP97A3 overexpression suggest a deficient CYP97A3 activity in CRC. Since sequence information for carrot cytochrome P450 hydroxylases is available for the ε-ring-specific CYP97A1 (Just et al., 2007), but not for CYP97A3, we first cloned CYP97A3 from QAL, assuming its intactness in white-rooted carrots. Based on sequence conservation of CYP97A3 homologs, we amplified a 1.3-kb fragment and completed the contiguous coding region by rapid amplification of cDNA ends (‘5’end) and genome walking (‘3’end). A phylogenetic analysis with the deduced amino acid sequence (616 amino acids) confirmed its identity as carrot CYP97A3 (Figure 5A). This was concluded from the high sequence identity to several cytochrome P450 enzymes, which were functionally confirmed as β-ring-specific α-carotene hydroxylases, e.g., from tomato (Sl-CYP97A29, 76% identity; Stigliani et al., 2011), rice (Os-CYP97A4, 69% identity; Quinlan et al., 2012), and Arabidopsis CYP97A3 (71% identity; alignment in Supplemental Figure 6). In contrast, the sequence exhibited only 41% identity to ε-ring-specific carotene hydroxylases, including carrot CYP97C1, and branched-off separately in phylogenetic analysis, excluding that the sequence obtained represented an additional CYP97C1 copy (Figure 5A). DNA gel blot analysis with genomic DNA from QAL and CRC revealed the presence of carrot CYP97A3 as single-copy gene in both cultivars. Quantitative RT-PCR (qRT-PCR) conducted on root RNA from QAL and CRC revealed no large differences in expression levels (Supplemental Figure 7), so that the possibility of mutations was considered.

An Insertion Present in a CYP97A3 Allele in Orange Carrots

Total leaf RNA was isolated from CRC and NAN plants and used to amplify CYP97A3 with the same primer combination used to amplify CYP97A3 from QAL. Remarkably, CYP97A3 cDNAs from both orange-rooted varieties were 8 nucleotides longer than from QAL, which was caused by a single insertion at position #1074 (Figure 5B; the electrophoretic mobility shift is shown in Supplemental Figure 7). The insertion results in a premature translational stop, encoding a truncated protein with only 382 instead of 616 amino acids (CYP97A3ins, Figure 5C). The truncated protein lacks a C-terminal cysteine residue that is essential for the assembly of the heme cofactor; it is therefore nonfunctional.

In order to confirm the involvement of the CYP97A3 gene in α-carotene accumulation in carrot roots, the identified insertion/deletion was genotyped in a broad unstructured population of 380 individuals, and an association mapping strategy was conducted on carotenoid content. Three associations were found to be significant with the insertion/deletion tested. The α-carotene content was significantly associated with this polymorphism, as expressed in dry (P value = 3.53 × 10⁻⁵) or fresh weight
P value = 5 \times 10^{-5} and explained 12 and 14% of the observed variation, respectively (Figure 5D; Supplemental Figure 8). Finally, this insertion was significantly associated with the α-β-carotene ratio (P value = 3.72 \times 10^{-4}), but explained a small proportion of the observed variation (r^2 = 0.04). Associations with any other carotenoid were not significant. These results confirm the involvement of CYP97A3 in α-carotene accumulation and explained a relatively high proportion of the variability of α-carotene content in carrot roots.

DISCUSSION

Cytochrome P450 Carotene Hydroxylase Deficiency in Orange Carrots

α-Carotene is the second most abundant carotene present in orange carrot cultivars. This is not limited to roots; α-carotene accumulates to unusually high amounts in leaves and is increased up to 10-fold compared with leaves of white-rooted cultivars. In this work, we addressed this widespread feature of orange-rooted carrots that is absent from white-rooted cultivars.

Leaf α-carotene content varies between different plant species and is also increased in response toward low light intensities (Matsubara et al., 2009). However, the constitutively high α-carotene levels in orange carrots point to a genetic alteration like in the Arabidopsis lut5 mutant, carrying a disruption within the cytochrome P450 carotene hydroxylase CYP97A3 (Kim and DellaPenna, 2006). In fact, the expression of the Arabidopsis CYP97A3 gene in CRC largely rescued leaf carotenoid patterns, approaching those of white-rooted carrots, regarding their low α-carotene content, low α/β-carotene ratio, and the complete absence of α-cryptoxanthin. Similarly, roots of CYP97A3-overexpressing CRC lines revealed carotenoid patterns matching over-expressing a bacterial phytoene synthase (Maass et al., 2009) as well as of PSY-overexpressing tissues from other crops, such as potato tubers (Solanum tuberosum) or cassava roots (Ducreux et al., 2005; Welsch et al., 2010). These observations corroborate that α-carotene abundance in orange carrots is not a concomitant of increased pathway flux by high PSY levels, but rather represents a particular property of orange carrots.
These findings are in agreement with our identification of a CYP97A3 allele in various orange carrot cultivars that carries an 8-nucleotide insertion and encodes a truncated protein that is most likely dysfunctional. An association study conducted on a large unstructured carrot population revealed that this polymorphism is associated with both α-carotene content and α-/β-carotene ratio in roots. More specifically, 88% of the individuals homozygous for CYP97A3Ins contained high α-carotene levels (above 2 mg g⁻¹ fresh weight), while this applied for only 47% of those individuals carrying the wild-type allele. Therefore, the CYP97A3 polymorphism identified explains a large proportion of the variation observed, even though there are other currently unknown factors affecting α-carotene levels in orange carrots. This is in agreement with a quantitative trait loci study, which estimated four genes as the minimum number of genes involved in α-carotene inheritance (Just et al., 2007). CYP97A3 is likely dysfunctional. An association study conducted on a large unstructured carrot population revealed that this polymorphism is associated with both α-carotene content and α-/β-carotene ratio in roots. More specifically, 88% of the individuals homozygous for CYP97A3Ins contained high α-carotene levels (above 2 mg g⁻¹ fresh weight), while this applied for only 47% of those individuals carrying the wild-type allele. Therefore, the CYP97A3 polymorphism identified explains a large proportion of the variation observed, even though there are other currently unknown factors affecting α-carotene levels in orange carrots. This is in agreement with a quantitative trait loci study, which estimated four genes as the minimum number of genes involved in α-carotene inheritance (Just et al., 2007). CYP97A3 is likely dysfunctional. An association study conducted on a large unstructured carrot population revealed that this polymorphism is associated with both α-carotene content and α-/β-carotene ratio in roots. More specifically, 88% of the individuals homozygous for CYP97A3Ins contained high α-carotene levels (above 2 mg g⁻¹ fresh weight), while this applied for only 47% of those individuals carrying the wild-type allele. Therefore, the CYP97A3 polymorphism identified explains a large proportion of the variation observed, even though there are other currently unknown factors affecting α-carotene levels in orange carrots. This is in agreement with a quantitative trait loci study, which estimated four genes as the minimum number of genes involved in α-carotene inheritance (Just et al., 2007). CYP97A3 is likely dysfunctional. An association study conducted on a large unstructured carrot population revealed that this polymorphism is associated with both α-carotene content and α-/β-carotene ratio in roots. More specifically, 88% of the individuals homozygous for CYP97A3Ins contained high α-carotene levels (above 2 mg g⁻¹ fresh weight), while this applied for only 47% of those individuals carrying the wild-type allele. Therefore, the CYP97A3 polymorphism identified explains a large proportion of the variation observed, even though there are other currently unknown factors affecting α-carotene levels in orange carrots. This is in agreement with a quantitative trait loci study, which estimated four genes as the minimum number of genes involved in α-carotene inheritance (Just et al., 2007).
Likewise, both LCYb-deficient maize mutants as well as LCYe-silenced potato tubers accumulated higher total carotenoid levels (Diretto et al., 2006; Bai et al., 2009). Furthermore, enhanced lycopene accumulation in the tomato ogc mutant is considered to be due to increased activity of earlier enzymes of the pathway in addition to reduced lycopene cyclization caused by a deficient fruit-specific lycopene cyclase (Ronen et al., 2000; Bramley, 2002). The induced changes in metabolite abundance entailed altered expression of carotenogenic enzymes, which in most cases included elevated PSY transcript levels. Furthermore, recent findings suggest the involvement of cis-carotene intermediates in the regulation of tomato fruit-specific PSY1 expression (Kachanovsky et al., 2012).

PSY levels/activity are key to carotenoid accumulation in non-green plant tissues (Ye et al., 2000; Lindgren et al., 2003; Ducreux et al., 2005; Welsch et al., 2010), including carrot roots (Santos et al., 2005; Maass et al., 2009). The context described here between CYP97A3 overexpression leading to reduced PSY protein levels suggests the presence of a negative feedback regulation, stemming from carotenoids downstream of α-carotene or metabolites thereof, acting on PSY levels. The difference with respect to the examples given above is that the expression of carrot PSY genes remained unchanged upon At-CYP97A3 overexpression in our study, while PSY protein levels were reduced. We therefore present an example of the modulation of PSY protein levels by carotenoid metabolites through mechanisms acting beyond transcription. Whether the feedback regulation at this level affects additional enzymes of the pathway cannot be conclusively answered as yet due to the lack of suitable antibodies. However, there are several examples in non-green tissues in which varying the rate of phytoene synthesis affects the total carotenoid content strongly while the carotenoid pattern is hardly affected (Paine et al., 2005; Maass et al., 2009). It is therefore conceivable that feedback-inhibited phytoene synthesis is sufficient to explain the reduced carotene amounts in At-CYP97A3-overexpressing carrots.

**Metabolite-Induced Feedback Regulation of PSY Protein Abundance**

In contrast to carotenoids, apocarotenoids are known to function as signaling molecules. In fact, it is reasonable to assume that strongly reduced α-carotene levels in At-CYP97A3 overexpressing CRC roots are caused by enhanced metabolism. However, this is contrasted by the lack of accordingly elevated β-ring hydroxylated α-carotene derivatives, i.e., zeinoxanthin or lutein, especially in older roots. The levels of these xanthophylls increased only...
slightly, and total xanthophyll levels remained at as low levels as in nontransformed CRC control plants. This suggests increased xanthophyll turnover through their enhanced cleavage by carotenoid cleavage oxygenases (Simkin et al., 2004; Rodrigo et al., 2013). In fact, several carotenoid-derived cleavage products are important signaling molecules involved in developmental processes (etiolactones) and mediate responses toward abiotic stress (ABA; Walter and Strack, 2011; Alder et al., 2012). However, the expression of the ABA-responsive carrot NCED3 was unchanged upon carotenoid changes in the transgenics. It therefore appears unlikely that ABA is involved in the observed response.

A putative function of other metabolites might be to trigger total carotenoid flux via the regulation of the rate-limiting steps of the pathway, most notably PSY. A dose dependence of PSY protein levels from carotenoid-derived metabolites might in fact be concluded from our data. α-Carotene levels in line Dc#25 are much lower than in line Dc#1, which might correlate with higher levels of cleavage products derived from ε-ring xanthophylls. Interestingly, this correlates with a more pronounced negative feedback of Dc#25 over Dc#1 since PSY protein levels are lower in Dc#25, corresponding with lower total carotenoid levels.

Similarly, an involvement of metabolites derived from ε-ring hydroxylated xanthophylls might also be concluded from transgenic tomato plants fruit-specifically overexpressing the bacterial desaturase crtI, which show an unexpected strong reduction in total carotenoid levels including phytene (Römer et al., 2000). While α-carotene is almost absent in control fruits, crtI-overexpressing fruits accumulate small amounts of α-carotene and ε-ring xanthophylls, which correlate with PSY enzyme activity reduced by half.

As shown previously, high abundance of carotenoids result in their crystallization, a mode of sequestration that reduces their accessibility toward degradation, either enzymatically or nonenzymatically (Maass et al., 2009; Nogueira et al., 2013). Therefore, the suggested negative feedback inhibition might also be part of a regulatory network that prevents critically high carotene concentrations in order to maintain carotenoid homeostasis.

It is currently unclear which of the two putative carrot PSY paralogs is affected by the negative feedback, as the antibody used cannot differentiate between these variants. Whether reduced PSY translation or increased protein turnover results in decreased steady state PSY protein levels cannot be conclusively answered yet. However, lower PSY protein levels in AtCYP97A3-overexpressing carrots do not entail higher levels of putative PSY breakdown products. Furthermore, PSY protein can be very strongly expressed in Arabidopsis (Maass et al., 2009), which may point to protein turnover being less relevant for flux adjustments. Metabolite-dependent regulation of translation required for the fine-tuning of biosynthetic efficiency appears more likely. Investigations to further discriminate between these possibilities are currently underway.

**METHODS**

**Plant Material and Growth**

Arabidopsis thaliana (ecotype Wassilewskija) seeds were grown aseptically on Murashige and Skoog medium under long-day conditions for 14 d. Seedlings were grown in liquid nitrogen immediately after harvest and stored at −70°C for further analysis. Arabidopsis roots were harvested from seedlings grown according to Hétu et al. (2005). Seeds from carrots (Daucus carota) were obtained as follows: Queen Anne’s Lace, Richters Herbs; Küttiger, Dreschflegel Saatgut; Nantaise, Freya; Chantenay Red Cored, B&T World Seeds. Carrot plants were grown in soil under long-day conditions. Roots of 8-, 12-, and 16-week-old carrot plants were removed from the soil, ground in liquid nitrogen, and stored at −70°C for further analysis.

**Carrot Transformation**

The At-CYP97A3 coding region was amplified by PCR with primers 5'-GGGCGTCTCAATGCTGCTTTCTCCTTCTTACT-3' and 5'-AAAGATGAAACGTTGAGAAAGAGGAGTGAACACTCATCC-3' from the RIKEN full-length cDNA clone RAFL09-10-L12, thereby introducing Ncol and Pml restriction sites, respectively. The fragment was digested with Ncol and Pml and ligated into the vector backbone obtained from the accordingly digested vector pCAMBIA-DJSS-GUS (Arango et al., 2010), revealing pCAMBIA-DJSS-AtCYP97A3. This vector was used to generate transgenic CRC lines as described by Maass et al. (2009), with the exception that 10 mg mL−1 hygromycin was used for selection of transgenic calli.

**Cloning of Dc-CYP97A3**

Sequence alignments with CYP97A3 sequences from various taxa were performed to identify conserved regions. Degenerated oligonucleotides with wobble nucleotides were designed: forward, ATT GCT/G TCT/C GGT/A/G/C GA/G C/T ATT CAC T/C/G GT, and reverse, TGA AAG TT/CT AA/CC GT/A C/G A/G A/T CCT CGA/T GG. Total QAL leaf RNA was reverse transcribed using the reverse primer and the verso cDNA kit (Thermo), followed by a PCR using both primers and Phusion polymerase (Thermo). Specific primers were designed from the partial CYP97A3 sequence obtained and the 3' end of the mRNA was retrieved using the 5'/3' RACE kit (Roche Diagnostics) and the forward primer 5'-ACATCGC-TGTCCCTCAAGGGTGGA-3' according to the manufacturer’s instructions. Sequence information of the 5’ mRNA end was retrieved by genome walking (GenomeWalker Universal Kit; Clontech) followed by exon predictions using the FGENESH algorithm available in Softberry (www.softberry.com). Full-length CYP97A3 coding regions were amplified using the primers forward 5'-CCACGACCACTGCTCCAAATGAGGC-3' and reverse 5'-GACAAT-TGGCTGCACATACAAAGAT-3'. Sequences used for phylogenetic analysis were selected according to Stigliani et al. (2011). Phylogenetic trees were reconstructed using the neighbor-joining method in MEGA5 (Tamura et al., 2011). The evolutionary distances were computed using the Poisson correction method and bootstrap test was selected with 1000 replicates. A member of family 86 of the CTP was chosen as an outgroup. For GenBank accession numbers in Figure 5A.
TaqMan Real-Time RT-PCR Assay

Total RNA was isolated using the plant RNA purification reagent (Invi- 
trogen Life Technologies). RNA purification, DNaseI digestion, and re-
time RT-PCR assays were performed as described (Welsch et al., 2008). 
Carrot PSY1, PSY2, CYP97A3, and nos 3’UTR (present in the At-CYP973 
transgene mRNA) expression was detected with 6FAM-labeled probes, 
while SYBR green was used for NCED3 expression analysis. For primers 
and probes, see Supplemental Table 3. For 18S rRNA quantification, 
the eukaryotic 18S rRNA endogenous control kit (Life Technologies) 
was used. The relative quantity of the transcripts was calculated using 
the comparative threshold cycle method (Livak and Schmittgen, 2001). 
Data were normalized first to the corresponding 18S rRNA levels and then 
expressed relative to a selected sample indicated.

Carotenoid Extraction and Quantification

Carotenoids were extracted using lyophilized plant materials (5 mg for 
leaves; 20 mg for carrot roots) and analyzed by HPLC as described 
(Welsch et al., 2008).

Immunoblot Analysis

Generation and affinity purification of antibodies directed against Arab-
idopsis PSY is described by Maass et al. (2009). Serum containing anti-
At-CYP97A3 antibodies was used in 1:5000 dilutions. Proteins 
were extracted with phenol as described (Welsch et al., 2007). After SDS-
PAGE, blotting onto polyvinylidene fluoride membranes (Carl Roth), and 
treatment with blocking solution (TBS containing 5% [w/v] milk powder), 
membranes were incubated with antibodies in PBS containing 0.1% (v/v) 
Tween 20 and 1% (w/v) milk powder. For detection, the ECL system (GE 
Healthcare) was used. Protein gel blots were stripped and reprobed with 
anti-actin antibodies (Sigma-Aldrich). Quantification of band intensities 
was performed with ImageJ (Abramoff et al., 2004).

Validation through Association Mapping Analysis

Polymorphism in CYP97A3 was tested in association mapping analysis in 
a broad unstructured population obtained by three intercrossing gen-
erations of a diversified panel of 67 carrot cultivars. A total of 380 in-
dividuals were randomly chosen at the third generation and were grown in 
field (Agrocampus Ouest, Angers, France) following standard agronomic 
practices. Carotenoid contents were quantified by HPLC with a modi-
fied procedure adapted from Clotault et al. (2008). CYP97A3 polymorphism 
was genotyped by KASP Assay technology (LG Genomics), along with 
92 single nucleotide polymorphisms (SNPs) spread all over the genome. 
Raw data on carotenoid content and CYP97A3 polymorphism are given in 
Supplemental Data Set 1. Association analysis (Hall et al., 2010) was 
performed using the TASSEL software (Bradbury et al., 2007). As re-
latedness between individuals can lead to false positive detection, the 
association tests were based on a mixed linear model in which a kinship 
matrix, estimated by identity by states calculated on SNP data set, was 
added as a covariable (Kang et al., 2008).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data 
libraries under the following accession numbers: JQ655297 (Dc-CYP97A3 
mRNA), JQ655298 (Dc-CYP97A3 gene, partial), DQ192196 (Dc-CYP97C1), 
ECC67393.1 (Os-CYP97C2), ECC74248.1 (Os-CYP97A4), NP_190881 
(At-CYP97C1), A1tg31800 (At-CYP97A3), ABC59096 (Mt-CYP97C10), 
ABB25656.1 (Mt-CYP97A10), ACJ5968 (SI-CYP97C11), ACJ25969 
(SI-CYP97A20), Al5g58860 (At-CYP98A1), XP_002512609.1 (Re-CYP97A), 
XP_002510427.1 (Re-CYP97C), and DQ192202 (Dc-NCED3).

Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** HPLC Chromatograms from Carrot and Arabidopsis Leaves.

**Supplemental Figure 2.** At-CYP97A3 Expression in Transgenic CRC.

**Supplemental Figure 3.** Carotenoid Patterns of DJ3S<sub>p-c</sub>-AtCYP97A3 CRC Lines.

**Supplemental Figure 4.** Dc-NCED3 Expression in Roots of DJ3S<sub>p-c</sub>-AtCYP97A3 Lines.

**Supplemental Figure 5.** Carotenoid and PSY Protein Levels in Arabidopsis and Carrot Roots.

**Supplemental Figure 6.** Alignment of Carrot CYP97A3 with Other Cytochrome p450 Proteins.

**Supplemental Figure 7.** Gene Copy Number and Expression of Carrot CYP97A3.

**Supplemental Figure 8.** Allelic Effects of CYP97A3 Polymorphism on α-Carotene Content and α/β-Carotene Ratio.

**Supplemental Table 1.** Carotenoids in Leaves from DJ3S<sub>p-c</sub>-AtCYP97A3 Carrots.

**Supplemental Table 2.** Detailed Carotenoid Amounts in Roots from At-CYP97A3-Overexpressing CRC Lines.

**Supplemental Table 3.** Primers and Probes Used for qRT-PCR.

**Supplemental Data Set 1.** Carotenoid Contents and CYP97A3 Genotype.

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**AUTHOR CONTRIBUTIONS**

R.W., P.B., and E.G. conceived this project and designed all experi-
E.G., and M.J. analyzed data. R.W., P.B., and E.G. wrote the article.

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Carotene Hydroxylase Activity Determines the Levels of Both α-Carotene and Total Carotenoids in Orange Carrots
Jacobo Arango, Matthieu Jourdan, Emmanuel Geoffriau, Peter Beyer and Ralf Welsch
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