Rice Carotenoid β-Ring Hydroxylase CYP97A4 is Involved in Lutein Biosynthesis

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Lutein is the most abundant plant carotenoid and plays essential roles in photosystem assembly and stabilization, as well as protection against photostress. To date, only a few lutein biosynthesis genes have been identified in crop plants. In this study, the rice Cyt P450 gene CYP97A4 encoding a carotenoid β-ring hydroxylase was shown to be involved in lutein biosynthesis. The results revealed that CYP97A4 was preferentially expressed in leaf compared with spikelet, sheath, stalk and root, and encoded a protein localized to the subcellular level to the chloroplasts. Compared with the wild type, the three allelic mutants of CYP97A4 displayed lutein reductions of 12–24% with substantially increased α-carotene, while Chl a/b levels were unaltered. The increased α-carotene in the mutants led to greater sensitivity under high light stress. Similarly, reactive oxygen species (ROS) imaging of leaves treated with intense light showed that the mutants generally accumulated greater levels of ROS compared with wild-type plants, which probably caused detrimental effects to the plant photosystem. In conclusion, this study demonstrated the important role of CYP97A4 in α-carotene hydroxylation in rice, and knock-out of the gene reduced lutein and increased α-carotene, contributing to sensitivity to intense light.

Keywords: Carotenoid hydroxylase • Cytochrome P450 • High light stress • Lutein • Rice.

Abbreviations: APCI-MS, atmospheric pressure chemical ionization mass spectrometry; BAC, bacterial artificial chromosome; BN-PAGE, blue native-PAGE; EMS, ethyl methanesulfonate; GUS, ß-glucuronidase; LHCII, light-harvesting complex II; NBT, nitroblue tetrazolium; NPQ, non-photochemical quenching; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; UTR, untranslated region; YFP, yellow fluorescent protein

Introduction

Carotenoids comprise a class of tetraterpenes prevalent in photosynthetic autotrophic plants and algae. Plant carotenoids are synthesized and accumulate in plastids and perform a variety of roles, including photosystem assembly and stabilization, auxiliary light harvesting, energy transmission and excess energy dissipation. Generally, carotenoids consist of carotenes and oxygenated forms of carotenes, xanthophylls. Lutein, a dihydroxy xanthophyll, is the predominant carotenoid in plant leaves, playing vital roles in light-harvesting complex II (LHCII), such as subunit assembly, photosystem stability maintenance and photoprotection (Bassi et al. 1993, Kuhlbrandt et al. 1994, Pogson et al. 1996, Cunningham and Gantt 1998, Yamamoto and Bassi 2004, Dall’Osto et al. 2006, Lokstein et al. 2002, DellaPenna and Pogson 2006). By electron crystallography, Kuhlbrandt et al. (1994) provided direct evidence that each LHCII monomer contains two molecules of lutein, with both polyene chains lying in a hydrophobic environment. This pair of lutein molecules forms a cross, providing an internal rigid linkage to strengthen the stability of LHCII. Two recent papers have presented with better clarity the structural details of the spatial relationships between Chls and lutein (Liu et al. 2004, Standfuss et al. 2005).

Through genetic studies of green algae and Arabidopsis single, double or triple mutants deficient in lutein (Niyogi et al. 1997, Pogson et al. 1998, Dall’Osto et al. 2006, Dall’Osto et al. 2007b), lutein has also been proven specifically and efficiently to quench 3Chl*. The green algae Chlamydomonas reinhardtii mutant lor1 (defective in lycopene ε-cyclase) deprived of lutein was shown to have significantly reduced non-photochemical quenching (NPQ) compared with the wild type (Niyogi et al. 1997). The Arabidopsis ortholog of lor1, lut2, lacks lutein production completely. Likewise, a delayed and reduced level of NPQ has been observed in the lut2 mutant (Pogson et al. 1998). Another equivalent ε-ring cyclase knock-out mutant in the Wassilewskija background, lut2.1, is completely devoid of lutein as well (Dall’Osto et al. 2006). Also, in the wild type a remarkable enhancement of resistance to photobleaching was observed in trimeric LHCII compared with the monomeric form (Dall’Osto et al. 2006). Direct measurements of 3Chl* quenching and 3Car* formation of lutein- and violaxanthin-containing monomeric Lhcb1
proteins have shown that recombinant proteins combining with lutein exhibited slower photobleaching than those binding violaxanthin (Formaggio et al. 2001, Dall’Osto et al. 2006). Lutein is considered to specialize in triplet Chl quenching, while β-xanthophylls are indispensable for reactive oxygen species (ROS) scavenging and qE activation (Dall’Osto et al. 2007b). In a screen of the Arabidopsis npq1 mutant to identify novel components of qE (Li et al. 2009), a suppressor designated as s21npq1 was found to be a leaky point mutant in the LCY-B locus containing twice as much lutein as the wild type or npq1. This increased amount of lutein probably accounts for the elevated NPQ and qE via substitution of zeaxanthin to de-excite 1'Chl* directly through formation of a lutein radical cation instead of a zeaxanthin radical cation, and thus restores NPQ to a higher level in the suppressor (Li et al. 2009). In general, the conservation of lutein lies not only in its ubiquity from green algae to higher plants, but also in its contribution to the protective mechanisms (Pogson et al. 1998, Niyogi 1999).

The initial step in plant carotenoid production is condensation of two geranyl geranyl pyrophosphate (GGPP) molecules into one phytoene. Phytoene is converted to lycopene through several steps of desaturation and isomerization. Biosynthesis of lutein begins with the cyclization of lycopene to form α-carotene, a process which requires both ε-cyclase and β-cyclase enzymes. The subsequent hydroxylation of the β-ring and ε-ring in α-carotene sequentially by corresponding hydroxylases results in formation of lutein in DellaPenna and Pogson 2006, Kim and DellaPenna 2006). Cyclization of lycopene marks two different branches of the xanthophyll biosynthesis pathway. The one branch forms β-carotene and oxygenated derivatives, while the other branch produces α-carotene and lutein. The ε-ring in the α-carotene branch is catalyzed by LCY-E in Arabidopsis. When LCY-E is combined with LCY-B, the cyclases work together effectively and synergistically convert lycopene to α-carotene ((β,ε-carotene), the precursor of lutein (Cunningham et al. 1996).

Hydroxylation of α-carotene (β,ε-carotene) at the 3C and 3’C positions of both rings generates lutein (β,ε-carotene-3,3’-dil). Since the chirality of the hydroxyl group on the ε-ring is opposite of that on the β-ring, different types of monooxygenases are presumed to be required for hydroxylation reactions (Cunningham and Gannt 1998). Thus far, four hydroxylases have been cloned and identified in Arabidopsis that function in this process. By using in vitro functional complementation and homologous screening, two non-heme ferredoxin-dependent di-iron monooxygenase cDNAs, CrTR-B1 and CrTR-B2, were cloned from both Arabidopsis and pepper fruit (Sun et al. 1996, Bouvier et al. 1998, Tian and DellaPenna 2001). B1 and B2 are functionally additive and complementary to each other. The third β-ring hydroxylase in Arabidopsis is a Cyt P450 monohydroxylase, CYP97A3 (Kim and DellaPenna 2006). In the presence of both functional B1 and B2, the cyp97a3 null mutant displays merely 18% lutein reduction compared with the wild type, suggesting significant redundancy between these proteins (Kim and DellaPenna 2006).

Additionally, two peaks of novel composition emerged in the HPLC profile of cyp97a3. Based on analyses of HPLC retention time and mass spectra plus UV-visible light absorption spectra, the major peak was identified as being ε-carotene, and the minor peak as being α-cryptoxanthin. The lut1 mutant was the first isolated from ethyl methanesulfonate (EMS)-induced populations to be defective in lutein biosynthesis, with 85% reduction in lutein vs. the wild type, and considerable accumulation of the monohydroxy precursor zeaxanthin. T-DNA knock-out mutants lut1-3 and lut1-4, in which hydroxylation function is disrupted, completely lack lutein biosynthesis, indicating that LUT1 is the only ε-ring hydroxylase in Arabidopsis (Pogson et al. 1996, Tian et al. 2004). The LUT1 primary sequence shares 52% amino acid identity with LUT5. According to the systematic nomenclature of Cyt P450, LUT1 was named CYP97C1 and LUT5 was named CYP97A3 (Chappell 1998, Tian et al. 2004). Phylogenetic analysis of the primary sequences of CYP97 proteins favors the hypothesis that a duplication of the ancestral CYP97 occurred before the higher plant–green algae split. The phenotypic difference between the slight sensitivity of cyp97c1 and the high sensitivity of cyp97a3 under strong light stress clearly indicates that an efficient α-carotene β-ring hydroxylation activity is strongly selected since it would greatly enhance the resistance to photobleaching. As a result, the same importation exhibited great susceptibility to high light stress compared with corresponding wild-type plants. CYP97A4 may provide another tool for improvement of rice resistance to an adverse environment (e.g. high light), and biofortification of provitamin A.

### Results

The lutein biosynthesis pathway in Arabidopsis has been clearly illustrated by forward genetics methods. Two crucial mutant loci, lut1 and lut2, generated by EMS induction were previously isolated (Pogson et al. 1996). The lut2 mutant was found to lack lutein and was identified to be defective in ε-ring cyclase activity, while the lut1 mutant has severely reduced lutein levels and increased xanthophyll cycle carotenoids; furthermore, the lutein precursor zeaxanthin is accumulated, which is below the detection limit in the wild type, indicating that lutein synthesis is blocked at the last step, ε-ring hydroxylation (Pogson et al. 1996). A homologous sequence search of LUT1
identified LUT5, and the loss of function of this protein was found to cause less reduction of lutein than in the lut1 mutant. \(\beta,\beta\)-Xanthophyll biosynthesis was also affected (Kim and DellaPenna 2006). Based on studies with these informative mutants and in vitro Escherichia coli enzyme activity assays, the primary carotenogenesis pathway in Arabidopsis initiated from lycopene is proposed in Fig. 1.

**Structure prediction and sequence analysis of CYP97A4 protein**

The ortholog of LUT5 in rice, CYP97A4, has proved effective in \(\beta\)-ring hydroxylation in functional complementation of bacteria accumulating \(\beta\)-carotene (Quinlan et al. 2007) and was used to construct a phylogenetic tree to study the evolution of carotene hydroxylases (Kim et al. 2009, Khurana et al. 2010). However, no work has been carried out thus far to analyze the function of CYP97A4 in vivo. To arrive at this destination, first we analyzed the primary sequence of CYP97A4. The deduced polypeptide consists of 643 amino acids, although the full-length cDNA contains >4,200 nucleotides distributed among 18 exons, and its sequence shares up to 63% identity with LUT5 (Fig. 2A). Similar to the case in Arabidopsis, there are three CYP97 subfamily members in rice, CYP97A4, CYP97B4 and CYP97C2, and, interestingly, CYP97B4 is comprised of two alternatively spliced isoforms, a full-length protein and an N-terminally truncated protein (Fig. 2A). The missing sequence is likely to include a chloroplast transit peptide and transmembrane helix. CYP97A4 is predicted in silico to be chloroplast targeted by TargetP (von Heijne et al. 2000). Further prediction by ChloroP 1.1 (Emanuelsson et al. 1999) indicated that the cleavage site of the transit peptide is at the 41st and 42nd amino acid (Emanuelsson et al. 2007). Structure and topology calculation by the HMMTOP server (Tusnády and Simon 1998) suggested a single transmembrane helix in the N-terminus (Tusnády and Simon 1998). In further analysis of this P450 protein, a consensus sequence of (A/G)x(D/E)(T/S) which shapes a threonine-containing pocket for binding oxygen was found (Chapple 1998), as indicated in Fig. 2A. In this heme-binding protein, the cysteine in the conserved signature sequence of the binding domain FxxGxxxCxG serves as the fifth ligand to the heme iron (Fig. 2A). The evolutionary relationships between rice and Arabidopsis CYP97 subfamily proteins are represented by an unrooted Neighbor–Joining phylogenetic tree based on the homologous protein alignment (Fig. 2B).

**CYP97A4 tissue expression pattern and protein sublocalization**

To determine in which tissues the protein functions, we examined CYP97A4 expression in various tissues from seedlings and mature rice plants by reverse transcription–PCR (RT–PCR). As expected, there was higher expression of the gene in seedling shoots than in roots (Fig. 3A, left panel). We also addressed the expression pattern of CYP97A4 in more detail with flowering rice plants. As shown in Fig. 3A (right panel), the CYP97A4 transcript was preferentially expressed in green tissues (i.e. leaf, sheath, stalk and spikelet), with the highest expression in leaf and the least in root. Quantitative results of CYP97A4 transcription in each tissue of Nipponbare obtained by real-time PCR are presented (Supplementary Fig. S1A, B), which are similar to the above. Furthermore, expression of other members of the CYP97 subfamily in the rice genome was analyzed. Like CYP97A4, CYP97C2 showed a differential expression level between the two tissues of seedling, whereas no difference between the two tissues was observed for both types of CYP97B4 transcripts (Supplementary Fig. S1C). In the mature plant, CYP97C2 again showed a pattern identical to

![Fig. 1 Simplified carotenoid biosynthesis pathways in higher plants. Solid arrows and compounds indicate the primary biosynthetic routes. Dashed arrows and gray-colored compounds indicate alternative auxiliary reactions. Steps affected by mutation of CYP97AG are marked by bold lines. Abbreviations for active enzymes of each step: eLCY, e-cyclase; bLCY, b-cyclase; bOHase, b-ring hydroxylase; eOHase, e-ring hydroxylase; ZE, zeaxanthin epoxidase; NXS, neoxanthin synthase.](https://academic.oup.com/pcp/article-abstract/53/6/987/1806812/Rice-Carotenoid-Ring-Hydroxylase-CYP97A4-is)
Fig. 2 Sequence alignment of predicted CYP97 subfamily proteins from rice (*Oryza sativa* ssp. *Japonica* var. Nipponbare) and *Arabidopsis thaliana* (ecotype Columbia). (A) ClustalW2 alignment of the rice and Arabidopsis CYP97 amino acid sequences. According to the conservation (continued)
CYP97A4, while both CYP97B4 isoforms did not reach such a high degree of consensus with each other and with CYP97A4 (Supplementary Fig. S1D).

Additionally, we examined CYP97A4 gene activity using transgenic rice plants carrying the CYP97A4 promoter–β-glucuronidase (GUS) fusion construct. Consistent with the result obtained by RT–PCR, GUS activity was mainly exhibited in the tissues such as leaf and sheath, while hardly any activity was detected in the root (Fig. 3B–E). In detail, green fluorescence signal from YFP (F, G top left), red autofluorescence signal from Chls (bottom left), bright field image (top right) and overlay of all signals (bottom right) are shown. Fluorescence was detected under a confocal laser scanning microscope. Bar = 5 μm.

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further corroborated the site where CYP97A4 would most probably be functionally active.

Isolation of cyp97a4 mutants and identification of CYP97A4 function

Previous *E. coli* functional complementation assays had demonstrated that CYP97A4 is capable of converting β-carotene into β-cryptoxanthin and zeaxanthin (Quinlan et al. 2007). Therefore, in order to clarify whether CYP97A4 plays the same role in vivo as in the heterologous *E. coli* expression system, we examined the relevant mutants selected from a database search, and three allele mutant lines were chosen from the Tos17 transposon insertion mutant database (Miyao et al. 2003, International Rice Genome Sequencing Project 2005). Each of the three mutants contains a Tos17 insertion in the exon region of CYP97A4. However, since 3–9 copies of Tos17 are located in the whole genome of each mutant, it was necessary and practical to find another corresponding wild-type control with a genome most resembling that of the mutant rather than the Nipponbare background control. Thus, the segregated wild-type plants from the mutant heterozygotes, designated 'TosWT', were chosen as homozygote controls. The CYP97A4 gene structure and Tos17 insertion sites are depicted in Fig. 4A, with the specific primer pairs indicated for RT–PCR amplification, the results of which showed that natural CYP97A4 mRNA was not detected in these homozygous insertion mutants (Fig. 4B). Although the CYP97A4 gene was disrupted in the cyp97a4-1, cyp97a4-2 and cyp97a4-3 mutant lines, no remarkable change in development or morphology was observed under normal growth conditions.

As the homologous protein of CYP97A4, LUT5 functions in lutein biosynthesis, specifically by encoding a hydroxylase, which primarily adds hydroxyl groups to β-rings of both α- and β-carotene. Lesions in LUTS have been shown to affect lutein production remarkably (Kim and DellaPenna 2006). In this study, the HPLC profiles of leaf extracts showed that all three allele mutants had reduced lutein content compared with both TosWT and Nipponbare controls (Table 1, Fig. 4C). cyp97a4-1 had a 20% reduction, while cyp97a4-2 and cyp97a4-3 showed 24 and 12% reduced levels, respectively, of lutein compared with TosWT (Fig. 4C). Neoxanthin, xanthophyll cycle carotenoids and β-carotene were also affected in at least two of the three mutant lines. As a result, total β,β-carotenoïd abundance in each cyp97a4 mutant was significantly different from that of the the TosWT.

Two novel peaks appeared in the HPLC elution profiles of the mutants compared with TosWT and Nipponbare controls (Fig. 4C). A minor peak (1) was found between Chl b and Chl a, during the metaphase of the elution process, while the major peak (2) was closely adjacent to and partially overlapped with β-carotene. As neither compound was affected by saponification (Supplementary Fig. S2A), the possibility of these peaks being carotenoid ester or Chl was ruled out. According to the retention time, peak (1) could have been a monohydroxy carotene derivative, including α-cryptoxanthin, β-cryptoxanthin and zeinoxanthin, due to its polarity being stronger than that of non-polar carotene but lower than that of dihydroxy carotenoids, e.g. lutein. The UV-visible absorption spectrum of peak (1) showed extraordinary similarity to that of α-carotene, but with some deviations from β-carotene in respect to the three maximum wavelengths of absorbance and the spectrum shape (Fig. 5A). Hence, β-cryptoxanthin was excluded as a carotenoid species for this peak. Finally, the identity of peak (1) as α-cryptoxanthin was confirmed by atmospheric pressure chemical ionization mass spectrometry (APCI-MS). The molecular ion of α-carotene (M+H)+ was at an m/z of 537 (Fig. 5C), while the major peak in the mass spectrum of lutein was at m/z 551, corresponding to H2O elimination (M+H-H2O)+. Additionally, no H2 adduct (M+H)+ with an m/z of 569 was detected (Fig. 5B), indicating that the hydroxyl group at C-3 of the ε-ring was readily removed (Pogson et al. 1996). The ionization profile of unknown peak (1) showed a main fragment ion at m/z 535, consistent with H2O elimination from the monohydroxy α-carotenoid (C40H56O). Furthermore, considering that α-cryptoxanthin eliminates H2O much more easily than zeinoxanthin due to an allylic hydroxyl group at C-3 of the ε-ring, we concluded that the minor peak (1) represented α-cryptoxanthin (Fig. 5B).

Judging from the retention time of peak (2), it could be a carotene with very similar polarity and structure to β-carotene. When the HPLC profiles from both the mutant and the mixed α,β-carotene standard were compared (Supplementary Fig. S2B), peak (2) was primarily presumed to be α-carotene. Our presumption was validated by the evidence that the UV-visible spectrum of the unknown peak (2) was identical with that of α-carotene (Fig. 5A), and that the main molecular ion in mass spectrum was at an m/z of 537, corresponding to the H2 adduct of carotene (Fig. 5C). Thus, we could safely conclude that peak (2) is α-carotene.

A genetic complementation test was next carried out to confirm the function of CYP97A4. An 11.6 kb DNA fragment from wild-type Nipponbare (background of tos17 mutants) genomic bacterial artificial chromosome (BAC) containing the CYP97A4 coding region and promoter region was transformed into cyp97a4 allelic mutants. The positive transgenic lines showed similar HPLC profiles and pigments levels to those of controls (Supplementary Fig. S3). These results indicated that CYP97A4 could restore the tos17 mutants to the wild-type phenotype. Taken together, these results indicated that CYP97A4 is a monooxygenase that specifically adds a hydroxyl group onto the β-ring of molecules in both branches of the pathway.

Composition and structural stability of thylakoid membrane pigment–protein complexes

Lutein deficiency mutants of Arabidopsis have been shown to have high levels of LHCl trimer disassembly and concomitant accumulation of LHClI monomers, while the
remaining fraction of trimers is approximately proportional to the lutein content of the residue (Lokstein et al. 2002, Kim and DellaPenna 2006). By using recombinant Lhcb1 protein synthesized in bacteria, it was revealed that not only mixed xanthophyll species but also lutein alone is sufficient for trimerization of LHCII, which cannot be substituted by violaxanthin (Dall’Osto et al. 2006). These results indicate that lutein is indispensable for assembly of LHCII trimers.

To explore whether reduced lutein in \( \text{cyp97a4} \) mutants would cause any structural alteration of the photosystem complexes, thylakoid membranes from mutants and controls were solubilized using mild detergent, and the pigment–protein complexes were subjected to Blue Native (BN)-PAGE analysis (Fig. 6). Consistent with previous reports, the LHCII trimer band for each of the mutants was lighter than that of the corresponding TosWTs, indicating a reduced stability of trimeric LHCII in the mutants. This reduction of LHCII trimers in the \( \text{cyp97a4} \) mutants basically corresponds to reduced lutein content. Additionally, the bands of LHCII–PSII supercomplexes seem to be affected as well. The relationship between the lutein level and supercomplex assembly had been observed previously in the rice \( \text{crtiso} \) mutant (Wei et al. 2010). We presumed that the decrease in supercomplexes may have resulted from the reduction of LHCII trimers, which is caused by the impaired lutein biosynthesis.
Thus lutein reduction and changes of other carotenoid species (Bassi et al. 1993, Kuhlbrandt et al. 1994, Pan et al. 2011). Lutein is the most abundant carotenoid in leaf tissue, accounting for half of the total carotenoids, and localizes in the LHCII center as well as in minor antenna to stabilize the complex and controls. Leaves (Fig. 7), there was no obvious difference in thylakoid membrane organization between cyp97a4-1 and controls. There were 5.9 ± 2.0 granum stacks in cyp97a4-1, 6.0 ± 1.9 in TosWT_a4-1 and 5.9 ± 2.0 in Nipponbare, respectively. All values are the mean ± SD. Statistical analyses confirmed that such an alteration of carotenoid composition, caused by null

### Table 1 Leaf tissue carotenoid composition (m mol/mol chl a + b) of seedlings of the listed genotype

<table>
<thead>
<tr>
<th>Line</th>
<th>Nipponbare</th>
<th>TosWT_a4-1</th>
<th>cyp97a4-1</th>
<th>TosWT_a4-2</th>
<th>cyp97a4-2</th>
<th>TosWT_a4-3</th>
<th>cyp97a4-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lutein</td>
<td>80.5 ± 3.7</td>
<td>84.5 ± 3.2</td>
<td>68.1 ± 5.1*</td>
<td>81.6 ± 1.3</td>
<td>62.2 ± 1.0*</td>
<td>79.4 ± 2.8</td>
<td>69.7 ± 2.8*</td>
</tr>
<tr>
<td>α-Cryptoxanthin</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.4</td>
<td>5.5 ± 0.4*</td>
<td>2.1 ± 1.3</td>
<td>5.8 ± 0.2*</td>
<td>2.6 ± 1.2</td>
<td>5.0 ± 0.2*</td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>27.5 ± 3.0</td>
<td>32.9 ± 1.3</td>
<td>29.6 ± 1.1*</td>
<td>27.8 ± 0.9</td>
<td>22.1 ± 1.0*</td>
<td>26.4 ± 3.1</td>
<td>25.4 ± 1.6</td>
</tr>
<tr>
<td>VAZ</td>
<td>66.3 ± 4.6</td>
<td>64.1 ± 1.6</td>
<td>59.9 ± 4.4</td>
<td>66.4 ± 1.6</td>
<td>51.4 ± 2.4*</td>
<td>67.8 ± 4.2</td>
<td>58.2 ± 3.0*</td>
</tr>
<tr>
<td>α-Carotene</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.4</td>
<td>20.8 ± 4.3*</td>
<td>2.1 ± 1.3</td>
<td>18.9 ± 1.5*</td>
<td>2.6 ± 1.2</td>
<td>16.7 ± 2.8*</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>65.6 ± 6.8</td>
<td>56.6 ± 10.2</td>
<td>48.3 ± 7.7</td>
<td>47.1 ± 1.9</td>
<td>40.8 ± 2.4*</td>
<td>54.7 ± 8.0</td>
<td>35.7 ± 5.1*</td>
</tr>
<tr>
<td>OH-β-ring</td>
<td>268.2 ± 15.3</td>
<td>278.5 ± 8.6</td>
<td>247.1 ± 12.9*</td>
<td>270.0 ± 5.8</td>
<td>209.2 ± 4.3*</td>
<td>267.9 ± 10.0</td>
<td>237.1 ± 9.1*</td>
</tr>
<tr>
<td>Xanthophyll</td>
<td>175.7 ± 9.5</td>
<td>182.8 ± 5.5</td>
<td>163.1 ± 8.0*</td>
<td>177.9 ± 3.0</td>
<td>141.5 ± 2.1*</td>
<td>176.2 ± 5.5</td>
<td>158.5 ± 5.5*</td>
</tr>
<tr>
<td>β,β-Carotenoids</td>
<td>159.4 ± 11.2</td>
<td>153.6 ± 9.7</td>
<td>137.8 ± 9.0*</td>
<td>141.3 ± 2.3</td>
<td>114.3 ± 2.9*</td>
<td>149.0 ± 7.9</td>
<td>119.3 ± 7.6*</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>242.6 ± 14.6</td>
<td>240.7 ± 8.3</td>
<td>232.1 ± 8.4</td>
<td>227.0 ± 4.5</td>
<td>201.1 ± 4.7*</td>
<td>233.5 ± 9.8</td>
<td>210.9 ± 12.9*</td>
</tr>
<tr>
<td>Chl a/b</td>
<td>2.57 ± 0.04</td>
<td>2.50 ± 0.03</td>
<td>2.57 ± 0.06</td>
<td>2.51 ± 0.03</td>
<td>2.50 ± 0.05</td>
<td>2.67 ± 0.05</td>
<td>2.62 ± 0.04</td>
</tr>
<tr>
<td>β,ɛ/β,β (cars)</td>
<td>0.52 ± 0.02</td>
<td>0.57 ± 0.05</td>
<td>0.69 ± 0.05*</td>
<td>0.61 ± 0.02</td>
<td>0.76 ± 0.02*</td>
<td>0.57 ± 0.02</td>
<td>0.77 ± 0.01*</td>
</tr>
</tbody>
</table>

Carotenoids and content are presented as millimoles of pigments per mole of total Chls. Values shown are means ± SD (n = 4). Student’s t-test was used for two samples. Values are not significantly different between Nipponbare and each TosWT except for the ratio of β,ɛ/β,β (cars) from the three TosWTs, as well as the values of β-carotene and β,β-carotenoids from TosWT_a4-2, which did not affect the significance of differences between each TosWT and cyp97a4 match. Those values marked with the symbols *, § and ↑ showed a significant difference between the TosWT and mutant, respectively.

VAZ, the total moles of violaxanthin, antheraxanthin and zeaxanthin; OH-β-ring, the millimoles of all hydroxylated β-rings per moles of Chl a + b; Chl a/b, the molar ratio of Chl a to Chl b; β,ɛ/β,β (cars), the molar ratio of β,ɛ-carotenoids to β,β-carotenoids.

**Ultrastructure of cyp97a4 chloroplasts**

Lutein is the most abundant carotenoid in leaf tissue, accounting for half of the total carotenoids, and localizes in the LHCII center as well as in minor antenna to stabilize the complex (Bassi et al. 1993, Kuhlbrandt et al. 1994, Pan et al. 2011). Thus lutein reduction and changes of other carotenoid species in cyp97a4 lines may cause structural alterations to the photosynthetic membranes and the organization of thylakoids in chloroplasts. In transmission electron microscopy analysis of ultrathin sections from 1-month-old mutants and controls leaves (Fig. 7), there was no obvious difference in thylakoid membrane organization between cyp97a4-1 and controls. There were 5.9 ± 2.0 granum stacks in cyp97a4-1, 6.0 ± 1.9 in TosWT_a4-1 and 5.9 ± 2.0 in Nipponbare, respectively. All values are the mean ± SD. Statistical analyses confirmed that such an alteration of carotenoid composition, caused by null
Physiological impact of CYP97A4 loss of function

Besides lutein and β-xanthophyll reduction in the lut5 mutant, accumulated α-carotene substitutes for β-carotene in the reaction center of the photosystem (Kim and DellaPenna 2006). α-Carotene has a smaller conjugated π-electron system than does β-carotene, and for this reason α-carotene is considered a less efficient photoprotectant, which may be useful for light harvesting to the leaves of shade-grown plants (Barth et al. 2001, Krause et al. 2001, Krause et al. 2004), but detrimental to the sun-grown plants. In agreement with this hypothesis, knock-out of LUT5 in Arabidopsis did lead to defective NPQ induction (Kim et al. 2009), and lut5 was shown to be fairly sensitive to strong illumination (Kim and DellaPenna 2006, Kim et al. 2009). Here, we tested cyp97a4 mutants and controls to clarify whether this phenomenon occurs in rice. Consistent with lut5, three cyp97a4 alleles uniformly displayed remarkable sensitivity to the high light stress (Fig. 8, upper panel). Seedling leaves in the mutants were photooxidized, while those of the controls remained in good condition.

Under high light irradiation, the absorbed light energy exceeds the photosynthetic utilization. As a result, excessive excitation energy leads to the production of too many ROS which exceed the capacity of detoxification. Thus, ROS should be primarily responsible for the photodamage (Niyogi 1999). To determine whether there was a different level of ROS between mutants and their respective controls, we examined the presence of superoxide with nitroblue tetrazolium (NBT) staining. As shown in Fig. 8 (mid and bottom panels), each cyp97a4 mutant has more intense staining than the corresponding TosWT and Nipponbare, although no difference was observed under normal conditions. These results indicated that CYP97A4 gene loss of function had led to the production of more superoxide, which may be caused by the different carotenoid levels, i.e. elevated α-carotene, and reduced β,β'-xanthophylls and lutein (Asada 1999, Niyogi 1999, Baroli et al. 2004, Dall’Osto et al. 2007a).

Discussion

The ubiquitous distribution of carotenoids, ranging from the lower archaea and cyanobacteria to higher plants and animals including humans, indicates conservatively fundamental yet diverse roles for various carotenoids in different organisms. Through molecular genetic and biochemical genomics-based approaches, carotenogenesis genes in the model organisms Arabidopsis and cyanobacteria, as well as other plants including tomato and pepper, have been cloned and characterized (Hirschberg 2001, DellaPenna and Pogson 2006). Thus, the primary carotenoid biosynthesis pathway has been established based on data acquired from informative single, double or triple mutants of those genes.

Although homologs of carotenogenesis genes in Poaceae were mentioned in some reports, only a few have been cloned and identified. Our results clearly showed that rice CYP97A4 was able to perform β-ring hydroxylation in vivo, a conserved function of this P450 subfamily. RT–PCR analysis revealed that expression of CYP97A4 was predominantly in shoots of rice seedlings and less in roots, which was confirmed by histochemical staining of promoter–GUS transgenic shoots of rice seedlings and less in roots, which was confirmed by histochemical staining of promoter–GUS transgenic seedlings showing that the CYP97A4 promoter was much more active in shoot than in root tissue. Analyses of mature rice plants revealed that CYP97A4 was preferentially expressed in green tissues such as leaf and sheath, while much lower transcript levels were detected in roots. The differential expression of CYP97A4 in different tissues, as well as prediction by TargetP and ChloroP that the first 41 amino acids serves as a chloroplast targeting signal, agreed with its important and distinct function in photosynthesis. Transient expression of the YFP-tagged transit peptide controlled by a 35S promoter showed that it exclusively accumulated in the chloroplasts. Thus, the subcellular localization of the protein further verified the silico prediction and the site where CYP97A4 carries out its hydroxylation activity. The deduced amino acid sequence of CYP97A4 contains two highly conserved motifs of the Cyt P450 enzyme as well, one for binding molecular oxygen and the other for binding heme. The evidence mentioned above suggested that CYP97A4 is a P450 protein localized in chloroplasts and should provide an important function for green tissues. However, no visible morphological change occurred in the
mutants vs. controls, and the Chl $\alpha/\beta$ ratio was also not affected.

Previous studies using a heterologous E. coli expression system had reported that the CYP97A4 protein is able effectively to add hydroxyl groups to both $\beta$-rings of $\beta,\beta$-carotene, but only exerts slight activity towards the $\varepsilon$-rings of $\varepsilon,\varepsilon$-carotene (Quinlan et al. 2007). In light of the inherent limitation of in vitro expression systems, including the lipid composition of the E. coli membrane where enzymes reside, it is necessary to identify the in planta function of CYP97A4.

In our study, HPLC analyses of related genotype plants revealed that depletion of rice CYP97A4 led to reduced $\beta$-ring hydroxylation in all three allelic mutants, and production of both $\beta,\varepsilon$- and $\beta,\beta$-xanthophylls was affected at different levels. The Arabidopsis lut5 mutant exhibits a similar reduction in lutein and $\beta,\beta$-carotenoid. Two intermediates, $\alpha$-cryptoxanthin and $\alpha$-carotene, are accumulated in lut5 (cyp97a3) due to P450 hydroxylase loss of function (Kim and DellaPenna 2006), which was also observed here in the rice cyp97a4 mutants. Thus, lutein biosynthesis apparently

![Transmisison electron micrographs of chloroplasts from leaf mesophyll cells of Nipponbare, isolated CYP97A4-1 control and mutant.](image)

**Fig. 7** Transmission electron micrographs of chloroplasts from leaf mesophyll cells of Nipponbare, isolated CYP97A4-1 control and mutant. (A, C, E) The chloroplast ultrastructures of cyp97a4-1, TosWT_a4-1 and Nipponbare, respectively. (B, D and F) The partial enlarged views of the respective graphs in A, C and E.
adheres to a conserved pathway in both dicotyledons and monocotyledons.

There is clear evidence that lutein is indispensable for trimerization of LHCII and rapid NPQ induction, and LHCII reconstitution in vitro has demonstrated that lutein alone is sufficient for LHCII trimerization (Lokstein et al. 2002, Dall'Osto et al. 2006). Thus, it was not surprising that partially impaired biosynthesis of lutein in *cyp97a4* mutants indeed resulted in reduced levels of LHCII trimers. Compared with controls, the elevated sensitivity of the *cyp97a4* mutants to high light stress may have been caused by the additive effect of a reduced level of lutein and β-xanthophylls. However, the striking accumulation of α-carotene, which was shown to be incorporated into photosystems instead of β-carotene in *lut5*, may be the most important factor responsible for the phenotype of the mutants under light stress. α-Carotene is mostly found in shade-grown plants, and it is likely that α-carotene performs better in light absorption than β-carotene under such an environment.

Fig. 8 Seedling leaf phenotypes of the respective rice genotype under high light stress. The upper panel shows the photodamage effect of in planta leaves from the three indicated mutant groups under normal illumination (200 μmol m⁻² s⁻¹; A, C, E) and after 1 d of high-light exposure (2,100 μmol m⁻² s⁻¹; B, D, F). The middle panel shows the images of detached seedling leaf (pre-infiltrated with NBT solution) of each indicated genotype under normal light (200 μmol m⁻² s⁻¹; G, I, K) or high-light treatment (900 μmol m⁻² s⁻¹; H, J, L) for 60 min. Images M, N and O in the bottom panel are enlarged views of the region marked by rectangles in H, J and L, respectively. Purple coloration indicates formation of formazon deposits produced upon NBT reactivity with superoxide.
(Thayer and Björkman 1990). However, benefit turns into weakness when the plants are exposed to a high light environment. α-Carotene-containing photosystems would produce more harmful singlet oxygen molecules and eventually damage the thylakoid membrane where photosynthesis occurs (Krause et al. 2001, Krause et al. 2004).

Important rice genes involved in lutein biosynthesis have been studied by forward genetics analyses of a series of related mutants. Due to lack of cyclization and resultant accumulation of lycopene, seed embryos of a rice homozygous lcy-b mutant take on a pink color, and mature seeds are prone to vivipary. Calli from lcy-b seeds, which initiate albino buds and develop albino shoots during tissue culture, die 4 weeks after germination (Fang et al. 2008). Another gene identified to participate in lutein biosynthesis is DSM2, a homolog of CrtRB in Arabidopsis. Knock-out of DSM2 was shown to lead to reduced ABA production under dehydration and enhanced drought and oxidative stress sensitivity. The feature distinguishing DSM2 from the other two CrtRB paralogs is the staggered developmental stage and tissue expression, which suggests specific and complementary functions of the family members (Du et al. 2010).

To understand the regulation of the CYP97A4 gene, analysis of its promoter region [2 kb upstream of the S’ untranslated region (UTR)] was carried out in the plantCARE websolver (Lescot et al. 2002). In addition to many cis-acting (regulatory) elements involved in light responsiveness, elements that respond to ABA and salicylic acid which may be related to biosynthesis and/or response to the hormones were also observed (data not shown). The presence of promoter elements that are involved in drought and heat stress, as well as defense and stress responsiveness, indicate that CYP97A4 takes part in regulation of photosynthesis efficiency under adverse conditions (Demming-Adams and Adams 1992). The effect of regulatory elements involved in circadian control was also detected. RT–PCR analyses of 24 h time-course samples showed that CYP97A4 expression was up-regulated about 10-fold during the night and decreased to normal levels the next morning (data not shown). The results are consistent with Arabidopsis LUT5 (CYP97A3) expression profiles (Pan et al. 2009). Interestingly, in spite of shifted phasing in the different branches, all carotenoid synthesis genes show circadian regulatory patterns. The lutein branch genes show phasing at 2 h before dark (14 h before next dawn), which is 5–6 h earlier than the zeaxanthin branch genes. Such a staggered and circadian-regulated accumulation of transcripts indicates that these branched pathways are controlled both separately and coordinately to maintain plant growth and fitness (Pan et al. 2009).

Investigations into lutein biosynthesis have found a chromatin-modifying histone methyltransferase, SDG8, which is necessary for transcription of an upstream carotenoid isomerase gene (CRTISO) (Cazzonelli et al. 2009). The study revealed that LCY-E is affected in a certain degree. LCY-E and LCY-B enzymatic activities constitute another important regulatory node to producing α-carotene and β-carotene in a modulated manner, and the flux distribution defines the abundance of downstream carotenoids in each branch (Cunningham et al. 1996, Pogson et al. 1996, Pogson and Rissler 2000, Li et al. 2009, Cazzonelli and Pogson 2010). LCY-E in maize displays many natural genetic variations for carotenoid composition (Harjes et al. 2008). Association mapping analysis revealed that four natural LCY-E polymorphisms can explain 58% of the variance in the α- and β-carotenoid and 3-fold differences in provitamin A compounds (including β-carotene, β-cryptoxanthin and α-carotene). Another example of a gene that can be targeted for provitamin A biofortification is maize crtRB1 (Yan et al. 2010). Association and linkage population studies demonstrated that crtRB1 defines a principal quantitative trait locus associated with β-carotene concentration and conversion in the kernels. The two loci mentioned above provide an efficient and inexpensive tool for provitamin A biofortification to combat dietary micronutrient deficiencies.

In conclusion, we identified and characterized a nuclear-encoded chloroplast Cyt P450 monoxygenase, CYP97A4, which is involved in β-ring hydroxylation and protection against high light exposure. Thus, genetic manipulation of CYP97A4 would be useful for enhancing resistance to adverse environments such as light stress and for improving provitamin content to increase the nutritional value of crops.

**Materials and Methods**

**Plant materials and growth conditions**

The three cyp97a4 alleles of rice tos17 insertion mutants (Oryza sativa L. ssp. japonica cv. Nipponbare) were obtained from the Rice Tos17 Insertion Mutant Database (Rice Genome Research Program; http://rgp.dna.affrc.go.jp/E/Analysis.html). The homogenous mutant and related wild-type control segregated from the heterozygous mutant were identified by PCR analyses with the given primer pairs flanking the tos17 insertion site and a primer on the tos17 sequence (Supplementary Table S1). The corresponding line names for the cyp97a4 alleles are: cyp97a4-1, NE5017; cyp97a4-2, NF6830; and cyp97a4-3, H0006. Seeds were placed in an incubator at 42 °C for 10 d to break the dormancy and then soaked in water at 30 °C for 3 d until germination. The uniformly germinated seeds were picked and sown in a 96-well plate in which the bottoms were removed. The plates were placed on a plastic box filled with water and transferred to the growth chamber (200 μmol m⁻² s⁻¹) with a 13 h daytime (26 °C)/11 h night-time photoperiod. Five days later the water was substituted with culture solution.

**High light stress and NBT staining**

For applying high light stress to leaves in planta, 30-day-old seedlings were transferred from normal light (200 μmol m⁻² s⁻¹) to the high light stress condition
(2,100 μmol m⁻² s⁻¹) for about 2 d following the regular photoperiod (2,100 μmol m⁻² s⁻¹ for 13 h, followed by 11 h in a dark chamber, and an additional 13 h of high light if needed). Leaves were affixed to a foam plate to keep the adaxial surface facing upward.

The seedling leaves were first excised at the petiole with scissors and allowed to be transpirationally imbibed in an aqueous solution of 6 mM NBT for 60 min in the chamber, and then transferred to the stress condition (900 μmol m⁻² s⁻¹) for 30–60 min. After stress, Chl was removed from the leaves with lacto-glycerol-ethanol (1:1:4 by vol.) and boiled in water for 5 min (Fryer et al. 2002). The purple formazan deposits indicated where NBT reacted with superoxide. The leaves were scanned, and images were saved.

**Sequence analyses and plasmid construction**

CYP97A4 cDNA and genomic sequence were analyzed mainly on the MSU Rice Genome Annotation Project Database (http://rice.plantbiology.msu.edu/) and Knowledge-based Oryza Molecular biological Encyclopedia (KOME) database (http://cdna01.dna.afrc.go.jp/cDNA/). The accession number of CYP97A4 in KOME is AK068163. The full-length cDNA was obtained from the RIKEN Institute (Carninci et al. 2000, Carninci et al. 2001). The P450-related information was acquired in the Cytochrome P450 database (http://drnelson.uthsc.edu/cytochromep450.html).

For construction of the complementation vector, the 11.6 kb DNA fragment, including an 8.3 kb CYP97A4 gene, 1.7 kb promoter fragment and 1.6 kb terminator region, was obtained by digestion of the Nipponbare genomic BAC clone OSJNBa0070K08 with BamHI alone. The BAC was acquired from the original developers of the BAC clones of the AGI (Arizona Genomics Institute) and NCGR (National Center of Gene Research, CAS). The 11.6 kb fragment was isolated from the agarose gel, sequenced and inserted into the BamHI-digested linearized pCAMBIA1301 vector. *Agrobacterium*-mediated transformation of the complementation construct into Nipponbare mutant calli was performed according to a standard protocol (Hiei et al. 1994).

**Analysis of CYP97A4 expression**

To identify the expression of CYP97A4 in mutants and control plants, as well as the expression pattern, total RNA was extracted from various tissues of Nipponbare seedlings and mature plants, and first-strand cDNA was reverse transcribed as described previously (Sun et al. 2009). RT–PCR and real-time qPCR were performed with the primers listed in Supplementary Table S1. For the analysis of CYP97A4 transcription with the GUS reporter system, the PCR-amplified 1.7 kb promoter region upstream of the 5′ UTR and together with the GUS reporter gene was inserted into a binary vector pCAMBIA1300 (Ren et al. 2005). The CYP97A4 promoter–GUS fusion construct was transformed to generate transgenic rice plants as described above.

**Subcellular localization of CYP97A4**

The first 300 bp cDNA sequence of CYP97A4 containing the transit peptide was cloned into the pA7-YFP vector. The CYP97A4 sequence was fused in-frame to the 5′ terminus of YFP (Tang et al. 2010). The translational fusion construct was transfected into tobacco protoplasts (Nicotiana tabacum), and the cells were incubated at 23°C overnight. Fluorescence of YFP was visualized with a confocal laser scanning microscope (Sheen 2001, Yoo et al. 2007).

**Leaf pigment analysis and quantification**

Leaf pigment was extracted in a microcentrifuge tube with approximately 10 mg of leaf tissue and 1 ml of 80% acetone. The leaves were cut into small pieces with scissors and soaked in the acetone solution. The tube was incubated at room temperature and periodically inverted at room temperature until the leaf tissue turned colorless. The mixture was centrifuged, and the supernatant was passed through a 0.45 μm filter. The extract was either stored at −20°C or analyzed by HPLC immediately according to the described methods (Tian and DellaPenna 2001). When needed, the extracts were saponified in 6% (w/v) KOH solution as previously described (Wurtzel et al. 2001), to remove Chl α and β, as well as the modification groups of carotenoid ester.

For quantification, four replicate plants per line were tested and analyzed. HPLC peak areas at 440 nm were integrated and calibrated by external standards. Chl α, Chl β, lutein, β-carotene and α,β-carotene mixed standards were purchased from Sigma-Aldrich Corporation to generate external calibration curves for quantification of the pigments (except α,β-carotene). Neoxanthin, violaxanthin, antheraxanthin, monohydroxyl α-carotene and α-carotene were estimated using the lutein curve (de Faria et al. 2009).

Chls and the known carotenoids were identified based on typical retention times and specific absorption spectra published in previous reports and comparison with authentic standards. For determination of the two unknown peaks accumulated in the cyp97a4 mutants, the saponified leaf pigment extracts were separated by HPLC, and the two specific peaks were separated, collected, dried down and resolubilized in a proper solvent. The purified and concentrated samples were submitted to the public laboratory for mass spectral analysis. Mass spectra were obtained using the Agilent 6520A Accurate-Mass QTOF LC-1200/MS (Agilent Technologies) with the Agilent ZORBAX Extend-C18 reversed-phase column, 3.0 × 50 mm, Φ = 1.8 μm. A 3 μl aliquot of concentrated sample was injected with a flow rate 0.3 ml min⁻¹. Mobile phase A was 71% acetonitrile/22% methanol (0.1 M ammonium acetate)/7% dichloromethane; buffer B was 20 mM ammonium acetate in acetonitrile. From 0 min, buffer B = 0% to 10 min 15%, 15 min 30%, 20 min 0% ending at 27 min. Other conditions were: DAD 440 nm, mass range 300–700, drying gas N₂ pressure 40 p.s.i., 350°C, 5 L min⁻¹; vaporizer...
420˚C; Vcap 3,800 V with an atmospheric pressure chemical ionization source operated in the positive ion mode.

**Thylakoid membrane preparation and BN-PAGE analysis**

Thylakoid membranes were prepared according to methods described previously (Zhou et al. 2009). The leaves were homogenized in an ice-cold isolation buffer containing 400 mM sucrose, 50 mM HEPES-KOH, pH 7.8, 10 mM NaCl and 2 mM MgCl₂ with a pre-chilled pestle and mortar and filtered through two layers of nylon cloth. After filtration, the thylakoids were centrifuged at 5,000 × g for 10 min, washed twice with isolation buffer and then resuspended in isolation buffer. The resulting thylakoid membranes were either used fresh or were frozen in liquid N₂ and stored at −70˚C before use. Thylakoid membrane complexes were separated by BN-PAGE as described (Schagger et al. 1994, Cline and Mori 2001). Each protein complex was identified as previously indicated (Wei et al. 2010).

**Transmission electron microscopy**

Wild-type and cyp97a4 leaves of 30-day-old seedlings were collected and cut into 1 mm squares, and fixed with EHTC14 in phosphate-buffered saline (PBS; pH 7.2) for 4 h at 4˚C. After fixation, the tissues were rinsed and post-fixed in 1% OsO₄ overnight at 4˚C. After rinsing in phosphate buffer, the samples were dehydrated in an ethanol series, infiltrated with a graded series of epoxy resin in epoxy propane and embedded in Epon 812 resin. Thin sections were stained in 2% uranyl acetate pH 5.0, followed by 10 mM lead citrate pH 12 and viewed with a transmission electron microscope, HITACHI H-7650 (Peng et al. 2006). To analyze the difference in the quantity of stacks per granum between controls and cyp97a4 mutant lines, 15 micrographs containing intact chloroplast from three independent plants per genotype were studied and used for statistical analysis with Student’s t-test.

**Supplementary data**

Supplementary data are available at PCP online.

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