Bottlenecks in carotenoid biosynthesis and accumulation in rice endosperm are influenced by the precursor–product balance

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Summary

The profile of secondary metabolites in plants reflects the balance of biosynthesis, degradation and storage, including the availability of precursors and products that affect the metabolic equilibrium. We investigated the impact of the precursor–product balance on the carotenoid pathway in the endosperm of intact rice plants because this tissue does not normally accumulate carotenoids, allowing us to control each component of the pathway. We generated transgenic plants expressing the maize phytoene synthase gene (ZmPSY1) and the bacterial phytoene desaturase gene (PaCRTI), which are sufficient to produce β-carotene in the presence of endogenous lycopene β-cyclase. We combined this mini-pathway with the Arabidopsis thaliana genes AtDXS (encoding 1-deoxy-D-xylulose 5-phosphate synthase, which supplies metabolic precursors) or AtOR (the ORANGE gene, which promotes the formation of a metabolic sink). Analysis of the resulting transgenic plants suggested that the supply of isoprenoid precursors from the MEP pathway is one of the key factors limiting carotenoid accumulation in the endosperm and that the overexpression of AtOR increased the accumulation of carotenoids in part by up-regulating a series of endogenous carotenogenic genes. The identification of metabolic bottlenecks in the pathway will help to refine strategies for the creation of engineered plants with specific carotenoid profiles.

Introduction

Plants synthesize a wide variety of natural products via complex secondary metabolic pathways (Miralpeix et al., 2013; Rischer et al., 2013). Many of the genes involved in secondary metabolism, transport and storage are poorly characterized, and the corresponding enzymes are often sequestered in subcellular compartments to add a further layer of complex regulation (O’Connor and Maresh, 2006). Therefore, many technical challenges must be overcome before multistep secondary metabolic pathways can be engineered effectively in heterologous plants.

The endosperm of cereal seeds is a major food staple, but it is deficient in many vitamins and minerals, including carotenoids (Zhu et al., 2007, 2008). Some carotenoids are beneficial but nonessential, for example those acting as antioxidants that help to prevent diseases such as cancer, whereas pro-vitamin A carotenoids such as β-carotene are regarded as essential nutrients because they cannot be synthesized de novo by humans (Bai et al., 2011; Fraser and Bramley, 2004; VonLintig and Vogt, 2004). Rice (Oryza sativa) is an important food staple in developing countries, but carotenoids do not accumulate in the endosperm, so its consumption as part of a nondiverse diet is often associated with vitamin A deficiency (Farre et al., 2010; Underwood and Arthur, 1996).

The carotenoid content and/or composition of staple crops can be enhanced by the expression of carotenogenic enzymes if isoprenoid precursors are available, and the carotenoid products do not accumulate to levels sufficient to oppose the equilibrium of the reaction. Upstream pathways supplying carotenoid precursors may therefore influence carotenoid accumulation (Rodríguez-Concepción, 2010), and downstream pathways that metabolize or sequester carotenoids to deplete the carotenoid pool can also drive the reaction forward (Auldridge et al., 2006; Campbell et al., 2010; Ohmiya et al., 2006). The naturally occurring dominant mutation Orange (OR) in cauliflower (Brassica oleracea cv. Botrytis) that induces proplastids and/or noncoloured plastids to differentiate into chromoplasts has been transferred to other crops to generate a metabolic sink that promotes the accumulation of carotenoids (Li and Van Eck, 2007; Lopez et al., 2008; Lu et al., 2006). Therefore, it should be possible to influence the rate of carotenoid synthesis in rice by regulating the supply of precursors and generating a metabolic sink to store the products (Cazzonelli and Pogson, 2010; Lu and Li, 2008).

The biosynthesis of carotenoids in rice endosperm is blocked at the first enzymatic step (phytoene synthase, PSY) which converts the precursor geranylgeranyl diphosphate (GGPP) into phytoene. There is also limited flux in the subsequent desaturation reaction, which generates lycopene. Strategies to boost carotenoid production in rice endosperm therefore involve the expression of heterologous PSY and the bacterial enzyme CRTI, which replaces several consecutive reactions that are required.
to produce lycopene in plants. Although this increases flux through the early part of the pathway, the expression of these two enzymes unmasks another rate-limiting step in the supply of precursors. For example, Golden Rice accumulates β-carotene by the endosperm-specific expression of maize (Zea mays) PSY (ZmPSY) and Pantoea ananatis CRTI (PaCRTI) under the control of the rice glutelin promoter, but it does not accumulate any phytoene, suggesting that the precursor pool is completely exhausted (Paine et al., 2005; Schaub et al., 2005; Ye et al., 2000).

To address this challenge, we expressed the heterologous ZmPSY and PaCRTI enzymes along with Arabidopsis thaliana 1-deoxy-D-xylulose-5-phosphate synthase (AtDXS) specifically in the endosperm to boost flux through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which generates carotenoid precursors (Figure 1). A metabolic sink was created in the endosperm by expressing the A. thaliana ORANGE (AtOR) gene (also under the control of an endosperm-specific promoter) which has recently been shown to sequester carotenoids in rice callus (Bai et al., 2014). It was found that AtDXS combined with ZmPSY1 and PaCRTI significantly enhanced the accumulation of carotenoids in rice endosperm, confirming that the supply of isoprenoid precursors such as GGPP is a rate-limiting step. The combined expression of ZmPSY1, PaCRTI and AtOR also boosted carotenoid accumulation through the creation of a metabolic sink, which resulted in the up-regulation of several endogenous carotenogenic genes. Our data provide a conceptual and mechanistic basis to resolve remaining bottlenecks in the carotenoid biosynthesis pathway in intact plants and will therefore facilitate the development of transgenic plants producing even higher levels of carotenoids in the endosperm.

**Results**

The combinatorial expression of carotenogenic genes in rice endosperm generates diverse genotypes with different carotenoid profiles

We transformed 7-day-old mature zygotic rice embryos with four constructs containing unlinked transgenes. Two of the genes encoded enzymes in the committed carotenoid biosynthesis pathway (ZmPSY1 and PaCRTI) and a third represented the MEP pathway (AtDXS). The fourth gene was the selectable marker hpt, which confers hygromycin resistance. The hpt gene was expressed constitutively, whereas AtDXS, ZmPSY1 and PaCRTI were controlled by the endosperm-specific rice RPS proliferin, wheat low-molecular-weight glutenin and barley α-hordein promoters, respectively. These experiments generated plants with different endosperm phenotypes (white, yellow or orange) depending on the combination of transgenes that were expressed (Figure 2). The behaviour of the transgenic lines was reproducible and consistent, that is all lines with the same complement of transgenes generated near-identical phenotypes.

We also investigated the consequences of sequestering carotenoids in a metabolic sink created by promoting chromoplast differentiation. To this end, we carried out a second set of experiments involving four transgenes, namely ZmPSY1, PaCRTI and the selectable marker hpt as above, but this time also the AtOR gene (controlled by the wheat low-molecular-weight glutenin promoter). These experiments also yielded transgenic rice plants with white, yellow or orange endosperm depending on the combination of transgenes that were expressed (Figures 2 and 3).

The analysis of steady-state mRNA levels showed that plants with yellow endosperm from both experiments expressed the ZmPSY1, PaCRTI and hpt genes, whereas those with the orange endosperm expressed the full complement of transgenes introduced in each experiment (Figure 3). Plants with white endosperm lacked ZmPSY1 and/or PaCRTI expression and were discarded because the phenotypes were not informative. More than 20 independent transgenic lines were generated with each gene combination that produced coloured grains, and from this pool, we selected three representative lines from each genotype to allow a more detailed comparison of metabolic profiles. Lines L1, L2 and L3 expressed ZmPSY1, PaCRTI and hpt only, and the total carotenoid content was 5.43, 5.51 and 4.61 μg/g dry weight (DW), respectively. Lines D1, D2 and D3 expressed the transgenes listed above plus AtDXS, and the total carotenoid content was 17.79, 14.94 and 31.78 μg/g DW, respectively. Lines O1, O2 and O3 also expressed the transgenes listed above plus AtOR, and the total carotenoid content was 11.53, 18.59 and

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**Figure 1** Reconstruction of the carotenoid biosynthesis pathway in rice endosperm (Bai et al., 2011; Farre et al., 2010). GA3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; AtDXS, Arabidopsis DXS; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; ZmPSY1, maize (Zea mays) phytoene synthase 1; PaCRTI, bacterial (Pantoea ananatis) phytoene desaturase; PDS, phytoene desaturase; ZISO, Z-carotene isomerase; ZDS, Z-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; ZISO, Z-carotene isomerase; ZDS, Z-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; CYP97C, carotene ε-ring hydroxylase; HYDβ, β-carotene hydroxylase (BCH, CYP97A or CYP97B); ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; ATOR, Arabidopsis ORANGE gene. L, seed endosperm expressing ZmPSY1 and PaCRTI; D, seed endosperm co-expressing AtDXS, ZmPSY1 and PaCRTI; O, seed endosperm co-expressing AtOR, ZmPSY1 and PaCRTI. Red circles indicate limiting enzymes numbered sequentially to reflect hierarchical bottlenecks in the pathway.
Figure 3. Transgenic expression analysis in rice endosperm. Relative expression of \( \Delta 8.1 \) and \( \Delta 10.8 \) enzymes in transgenic rice lines. (A) Total RNA was extracted from transgenic and wild-type rice lines. (B) The expression levels of \( \Delta 8.1 \) and \( \Delta 10.8 \) enzymes were quantified using real-time qRT-PCR. The expression levels of \( \Delta 8.1 \) and \( \Delta 10.8 \) enzymes were normalized to the expression levels of \( \Delta 8.1 \) and \( \Delta 10.8 \) enzymes in wild-type rice lines. The expression levels of \( \Delta 8.1 \) and \( \Delta 10.8 \) enzymes were calculated using the Livak method.

Table 1. Carotenoid content and composition of T3 endosperm at 40 DAP

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>O1</th>
<th>O2</th>
<th>O3</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
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<tr>
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<td>0</td>
<td>0</td>
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<tr>
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<td>0.25</td>
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<td>0.01</td>
<td>0.17</td>
<td>0.13</td>
<td>0.28</td>
<td>0.32</td>
<td>0.06</td>
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<tr>
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<td>0</td>
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<td>0.19</td>
<td>0.29</td>
<td>0.57</td>
<td>0.13</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>1.48</td>
<td>2.15</td>
<td>0.09</td>
<td>1.17</td>
<td>0.19</td>
<td>0.87</td>
<td>1.36</td>
<td>0.88</td>
<td>0.57</td>
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<td>Lutein</td>
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<td>0.26</td>
<td>0.02</td>
<td>0.26</td>
<td>0.14</td>
<td>0.58</td>
<td>1.36</td>
<td>0.88</td>
<td>0.57</td>
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<td>0.01</td>
<td>0.01</td>
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<td>0.42</td>
<td>1.41</td>
<td>0.90</td>
<td>0.42</td>
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<tr>
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<td>0.16</td>
<td>0.01</td>
<td>0.70</td>
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<td>9.70</td>
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<td>Phytolene</td>
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<td>2.05</td>
<td>0.01</td>
<td>2.05</td>
<td>1.57</td>
<td>1.57</td>
<td>0.42</td>
</tr>
<tr>
<td>Total Carotenoids</td>
<td>5.43</td>
<td>5.51</td>
<td>0.16</td>
<td>4.61</td>
<td>0.81</td>
<td>11.53</td>
<td>18.59</td>
<td>16.61</td>
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<tr>
<td>( % ) β-Carotene</td>
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<td>0.81</td>
<td>1.08</td>
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<td>1.42</td>
<td>1.50</td>
<td>40.73</td>
<td>47.92</td>
<td>57.77</td>
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</table>

Data are means ± SD from analysis of three independent seed batches (40 dap) and are expressed as μg/g DW. \( \% \) β-Carotene, the ratio of β-carotenoids to \( \% \) carotenoids; \( \% \) β-Carotene, the ratio of β-carotene to the total carotenoid amount (%). Rice endosperm expressing hpt has no detectable carotenoids. O, seed endosperm expressing ZmPSY1, PaCRTI and AtOR; D, seed endosperm expressing ZmPSY1, PaCRTI and ADXS; L, seed endosperm expressing ZmPSY1 and PaCRTI.
endogenous phytoene desaturase (OsPDS), lycopene ε-cyclase (OsLYCE), lycopene β-cyclase (OsLYCB), β-carotene hydroxylase (OsBCCH2) and zeaxanthin epoxidase (OsZEP) mRNAs by quantitative real-time PCR at the same time points (Figure 5). All five endogenous genes were expressed at similar levels in genotype L and the hpt-only control, indicating the absence of any feedback regulation in response to the expression of ZmPSY1 and PaCRTI. However, OsPDS was up-regulated in all three lines of genotype D and also in one of the lines expressing AtOR (line O3). There was no induction of OsPDS mRNA in lines O1 and O2, perhaps reflecting the lower level of AtOR expression in these lines compared to O3 (Figure 5).

The OsLYCE gene was strongly up-regulated in genotypes D and O compared to L. The OsLYCB gene was expressed at similar levels in genotypes L and D and the hpt-only control, but was induced 1.4- to 1.8-fold in genotype O. OsBCCH2 expression was suppressed in lines D1 and D2 but not in line O3, and the same gene was up-regulated in all three lines of genotype O (Figure 5). Finally, OsZEP mRNA levels were up-regulated by up to twofold in lines O1 and O2 but not in any other lines including O3, which may again reflect the much higher levels of AtOR mRNA in line O3 compared to O1 and O2 (Figure 5).

**Discussion**

Combinatorial transgenic expression results in diverse phenotypes reflecting the accumulation of different types and levels of carotenoids in the endosperm

Wild-type rice endosperm does not naturally produce carotenoids, and previous research has shown that significant levels of carotenoids can only accumulate in this tissue if a heterologous mini-pathway is imported representing all committed steps prior to the reaction catalyzed by lycopene β-cyclase. A heterologous daffodil (Narcissus pseudonarcissus) PSY gene expressed in rice resulted in the accumulation of phytoene, but no desaturated intermediates earlier in the pathway, and they are notable for the predominant production of phytoene, the intermediate product of PSY1 (Paine et al., 2005; Ye et al., 2000). These results suggest that there is an additional bottleneck upstream of the first committed pathway step, which means that the pathway precursors are almost entirely depleted. Furthermore, the build-up of β-carotene is likely to affect the equilibrium in the cell and inhibit further conversion, suggesting that β-carotene levels could be boosted further by increasing the precursor supply and removing the product by transferring it to a metabolic sink. In an earlier study, we therefore used our callus platform for the rapid functional characterization of genes to test the expression of AtDXS (to increase flux through the MEP pathway and therefore increase the availability of precursors for carotenoid synthesis) and AtOR, which induces the differentiation of chromoplasts and thus promotes the accumulation of carotenoids in the plastid (Bai et al., 2014). Surprisingly, we found that chromoplast differentiation could be induced directly not only by the wild-type AtOR gene but also by increasing the supply of precursors in the absence of AtOR (Bai et al., 2014).

Although the callus system is a useful in vitro platform, it is unlikely to represent accurately the intracellular milieu present in differentiated endosperm tissue. We therefore used our established combinatorial expression platform that allows the recovery of diverse metabolic libraries based on random combinations of input transgenes (Zhu et al., 2008) to produce transgenic lines expressing ZmPSY1 and PaCRTI (genotype L) complemented with either AtDXS (genotype D) or AtOR (genotype O). All the genotype L plants produced yellow grains reflecting the accumulation of carotenoids in the endosperm, and likewise the genotype D and genotype O plants produced orange grains. Other transgenic lines were recovered expressing either AtDXS or AtOR in the absence of ZmPSY1 and PaCRTI, but because these plants lacked essential steps in the early carotenoid synthesis pathway, they did not accumulate any carotenoids and the grains were white. Lines expressing only the selectable marker gene hpt were also recovered, and these were used as controls together with the lines expressing only AtDXS or AtOR.

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Increasing the precursor supply enhances carotenoid accumulation in rice endosperm and modulates endogenous carotenogenic genes

Carotenoids are synthesized primarily from precursors derived from the MEP pathway shown in Figure 1 (Eisenreich et al., 2001; Farre et al., 2010; Rodríguez-Concepción, 2010; Rodríguez-Concepción and Boronat, 2002). This pathway uses pyruvate and glyceraldehyde-3-phosphate to produce 1-deoxy-D-xylulose-5-phosphate (DXP) in a reaction catalyzed by DXP synthase (DXS). DXS is the rate-limiting step in the formation of plastid-derived isoprenoids (Enfissi et al., 2005; Estévez et al., 2001; Morris et al., 2006; Rodríguez-Concepción, 2010). Therefore, the fruit-specific overexpression of Escherichia coli DXS in tomatoes increased the total carotenoid content by up to 1.6-fold although most of this accumulated as phytoene and the end products lycopene and \( \beta \)-carotene were not boosted at all, suggesting that phytoene desaturation was a new rate-limiting step in the transgenic plants (Enfissi et al., 2005). Similar results were achieved in transgenic potatoes expressing E. coli DXS (Morris et al., 2006).

To determine whether DXS can enhance the isoprenoid precursor pool in rice endosperm, we compared transgenic rice lines co-expressing AtDXS, ZmPSY1 and PaCRTI (genotype D) with those co-expressing ZmPSY1 and PaCRTI (genotype L). The

Figure 5  Expression of endogenous carotenogenic genes in transgenic rice endosperm. Relative transcript levels of endogenous OsPDS, OsLYCE, OsLYCB, OsBCH2 and OsZEP genes in T3 rice endosperm at 25 dap expressing different transgenic combinations. Values are means ± SD of three quantitative real-time PCR replicates. The expression for each gene was normalized against actin mRNA. Abbreviations: PDS, phytoene desaturase; LYCE, lycopene \( \epsilon \)-cyclase; LYCB, lycopene \( \beta \)-cyclase; BCH, \( \beta \)-carotene hydroxylase; ZEP, zeaxanthin epoxidase. Line names are defined in the legend to Figure 3.
endosperm in genotype D was orange compared to the yellow endosperm in genotype L, indicating a qualitative and/or quantitative difference in carotenoid levels. Accordingly, we found that the three lines of genotype D accumulated 2.7- to 5.8-fold more total carotenoids than genotype L (Table 1). This was a greater increase than achieved with the same gene combination in callus (Bai et al., 2014), which confirmed that although the callus system is useful for rapid functional characterization, it cannot represent the more complex environment of the fully differentiated endosperm. In contrast to the tomato and potato lines described above (Enfissi et al., 2005; Morris et al., 2006), the levels of phytoene in rice genotypes D and L were similar, with averages of 1.5 and 1.7 µg/g DW, respectively (Table 1); thus, the higher carotenoid levels in genotype D mainly reflected increases in the levels of β-carotene, α-cryptoxanthin and α-carotene (Table 1). This correlates well with the observed up-regulation of endogenous phytoene desaturase (OsPDS) and lycopene ε-cyclase (OsLYCE) in these lines (Figure 5) because the combination of heterologous DXS and endogenous and heterologous PDS would increase flux through the entire pathway, whereas LYCE would specifically boost the production of α-carotene and α-cryptoxanthin (without the loss of β-carotene given the overall increase in flux caused by PDS activity). In contrast, the unaltered LYCB and BCH2 activity may give rise to a new bottleneck in response to the increased flux, thus resulting in the accumulation of β-cryptoxanthin prior to its conversion into beta-carotene. The total carotenoid content of endosperm expressing ZmPSY1, PaCRTI and AtDXS was at least threefold higher than that expressing only ZmPSY1 and PaCRTI (Table 1, Figure 4), indicating the supply of isoprenoid precursors such as GGPP derived from the MEP pathway is critical for maximizing carotenoid accumulation in rice endosperm. In the callus system, expression of the same three genes resulted mainly in the accumulation of β-carotene, α-carotene and phytoene, again reflecting differences between callus and endosperm in terms of the relative levels of precursors, enzymes and intermediates (Bai et al., 2014).

Creating a metabolic sink enhances carotenoid accumulation in rice endosperm and modulates endogenous carotenogenic genes

The cauliflower Or gene is the only known gene that acts as a bona fide molecular switch to trigger the differentiation of noncoloured plastids into chromoplasts (Giuliano and Diretto, 2007; Lu et al., 2006). It was discovered as a spontaneous dominant mutation in cauliflower, which caused minimally pigmented tissues such as the edible curd to become orange due to the accumulation of high levels of β-carotene (Li et al., 2001; Lu et al., 2006). The expression of cauliflower Or in transgenic potato tubers caused the ectopic induction of chromoplasts, allowing the tubers to accumulate substantial amounts of β-carotene, violaxanthin, lutein, phytoene, phytofluene and ζ-carotene (Lopez et al., 2008). There was no indication in these experiments that the cauliflower Or induced the expression of carotenogenic genes (Li et al., 2001, 2006; Lu et al., 2006). However, the overexpression of a sweet potato OR orthologue was recently shown to induce carotenogenic gene expression in transgenic sweet potato callus, suggesting that OR might promote carotenoid accumulation to the extent that chromoplast differentiation is triggered by the presence of excess carotenoids rather than the direct activity of the OR protein (Kim et al., 2013).

Recently, we demonstrated that the overexpression of the wild-type AtOR gene induced chromoplast formation in rice callus and enhanced carotenoid levels by forming a metabolic sink (Bai et al., 2014). However, the results from our analysis of the genotype L and genotype O transgenic plants described herein present a more complex picture, in which AtOR expression may influence carotenoid levels both directly and indirectly. The genotype O transgenic lines accumulated 2.1- to 4.7-fold more total carotenoids in the endosperm than genotype L, and most of this increase was accounted for by the 5.2-fold increase in the levels of β-carotene, β-cryptoxanthin and α-carotene, as well as a 2.1- to 6.6-fold increase in the levels of lutein (Table 1). This correlated well with the observed induction of the endogenous OsLYCE, OsLYCB and OsBCH2 genes and confirmed that AtOR promotes the modulation of endogenous carotenogenic gene expression in rice (Figure 5). The role of AtOR in chromoplast differentiation appears to be more complex than initially envisaged, because we noted in earlier experiments the formation of chromoplasts in rice callus of genotype O (expressing AtOR and the carotenoid mini-pathway) and genotype D (expressing AtDXS and the carotenoid mini-pathway but not AtOR), but we did not find any evidence for chromoplast differentiation in the endosperm of plants expressing AtOR in the absence of ZmPSY1 and PaCRTI. Transmission electron microscopy revealed a few electron-dense plastoglobuli (which may contain pigment) inside some plastids of 40 dap endosperm in the genotypes O and D, and various small plastoglobuli inside some plastids were visible in lines L, whereas no plastoglobuli were visible in lines HPT (expressing only the selectable marker gene hpt) (Figure 6). This suggests that chromoplast differentiation is primarily triggered by carotenoid accumulation above a certain threshold and that the presence of the Orange protein may augment or potentiate this process but is not sufficient without other drivers of carotenoid accumulation (Bai et al., 2014; Maass et al., 2009). Moreover, perturbations in carotenoid composition of Psy-1 transgenic tomato induced plastid differentiation during fruit development (Fraser et al., 2007).

AtDXS and AtOR regulate carotenoid biosynthesis in rice endosperm through distinct mechanisms

Our experiments revealed that AtDXS and AtOR regulate different sets of endogenous carotenogenic genes, suggesting they boost carotenoid accumulation via distinct mechanisms. In genotype O lines expressing AtOR, we observed the up-regulation of OsLYCE, OsLYCB, OsBCH2 and OsZEP, and only OsPDS was slightly affected (Figure 5). In contrast, in genotype D lines expressing AtDXS, we observed the up-regulation of OsPDS and OsLYCE but not the other three genes. Both AtDXS and AtOR thus boosted the accumulation of total carotenoids in rice endosperm by at least twofold when expressed in concert with ZmPSY1 and PaCRTI (Table 1). In both cases, this predominantly reflected the presence of higher levels of β-carotene and α-carotene (Table 1 and Figure 4). The level of endogenous OsLYCB increased in lines expressing AtOR but not AtDXS (Figure 5), suggesting endogenous OsLYCB is sufficient for enhanced β-carotene synthesis in lines overexpressing DXS as might be expected given the greater flux throughout the entire pathway. The level of endogenous OsLYCE mRNA was significantly up-regulated in lines expressing either AtDXS or AtOR (Figure 5), which explains the fivefold increase in the accumulation of α-carotene (Table 1).
Zeaxanthin and lutein levels were higher in the lines expressing AtOR, which was consistent with the induction of endogenous OsBCH2 expression (Table 1 and Figure 5). However, similar zeaxanthin and lutein levels were observed in the lines expressing AtDXS, in which endogenous OsBCH2 expression remained at normal levels (Table 1 and Figure 5). This argues that the flux increased in both lines, in one case due to the greater abundance of GGPP and in the other by the removal of a bottleneck at the hydroxylation step, reflecting the stronger expression of OsBCH2.

Phytoene levels were lower in genotype O (average 1.1 µg/g DW) than genotype D (average 1.5 µg/g DW) or genotype L (average 1.7 µg/g DW) (Table 1). Nevertheless, phytoene accumulated in all nine lines, suggesting that CRTI is not efficient enough to convert all phytoene into downstream carotenoids, which was not the case for Golden Rice (Paine et al., 2005). Phytoene was also detected in the plastoglobules of transgenic tomato fruit chromoplasts, whereas the corresponding enzymes are localized in the membranes (Nogueira et al., 2013). It is possible that phytoene and CRTI may also be compartmentalized separately in transgenic rice endosperm, resulting in residual phytoene accumulation, highlighting the important role of compartmentalization in the control of endogenous and introduced metabolic pathways.

A variety of factors determine the qualitative and quantitative profile of carotenoids in rice endosperm

The discordance between the endosperm carotenoid profiles in our rice lines and the previously described Golden Rice and Golden Rice II lines is noteworthy. One factor that may play a significant role is the cultivar, which was Kaybonnet in the case of Golden Rice II and EY1105 in our experiments, because the level of endogenous MEP and carotenoid pathway gene expression may differ (Paine et al., 2005). This may explain why the endosperm carotenoid composition of our lines was 25–39% β-carotene and 9–11% α-cryptoxanthin (Table 1), whereas in Golden Rice II, the equivalent values were 75–84% β-carotene and α-cryptoxanthin was not detectable (Paine et al., 2005). This suggests cultivar-specific differences in the endogenous β-carotene and α-carotene hydroxylase activities, consistent with the natural variation in the expression of carotenogenic genes not only in different varieties of rice but also in other cereals such as maize (Harjes et al., 2008) and sorghum (Kean et al., 2007).

Other differences may reflect the control of transgenic expression. We used the wheat low-molecular-weight glutenin promoter to control ZmPSY1 and the barley D-hordein promoter to control PaCRTI, whereas in Golden Rice II, both transgenes

Figure 6 Transmission electron micrographs of transgenic rice endosperm expressing ZmPSY1, PaCRTI and AtOR (a), ZmPSY1, PaCRTI and AtDXS (b), ZmPSY1 and PaCRTI (c), HPT (d). Arrows indicate plastoglobuli inside plastids (pl). Scale bar 0.3 µm (a, b and c) and 1 µm (d).
were controlled by the rice glutelin promoter (Paine et al., 2005). We have previously noted that different endosperm-specific promoters affect the mRNA and protein levels of the corresponding enzymes and also the level of any metabolites in the engineered pathway (Naqi et al., unpublished).

The rate of carotenoid degradation or turnover inversely determines carotenoid content (Nisar et al., 2015). A family of carotenoid cleavage dioxygenases (CCDs) catabolizes specific enzymatic turnover of carotenoids into apocarotenoids in maize, rice and sorghum (Vallabhaneni et al., 2010). The expression of carotenoid cleavage dioxygenase 1 was inversely correlated with the accumulation of carotenoids in maize endosperm (da Silva Messias et al., 2014; Vallabhaneni et al., 2010). The activities of various carotenoid cleavage dioxygenases might be different in Kaybonnet in the case of Golden Rice II and EY110S in our experiments. Our results clearly demonstrate a mechanistic basis for differences in carotenoid profiles between plants with different transgenic complements and genetic backgrounds, and also significant differences between the differentiated endosperm of intact plants and the in vitro screening system based on rice callus (Bai et al., 2014).

**Conclusions**

We have investigated factors that limit the accumulation of carotenoids in rice endosperm, specifically focusing on the precursor pool and the impact of a metabolic sink. The metabolic comparison of endosperm from genotype L plants (ZmPSY1 and PaCRTI) and genotype D plants (ZmPSY1, PaCRTI and AtDXS) confirmed that the supply of isoprenoid precursors from the MEP pathway is a key bottleneck, which limits flux through the entire pathway. The metabolic comparison of genotype L plants and genotype O plants (ZmPSY1, PaCRTI and AtOR) clearly demonstrated that the overexpression of AtOR can boost carotenoid accumulation in rice endosperm by creating a metabolic sink that drives the equilibrium of the pathway towards completion, and also by the up-regulation of endogenous carotenogenic genes. The twofold increase in total carotenoids achieved in genotypes D and O predominantly reflected the accumulation of provitamin A carotenoids to at least fivefold normal levels, based on the combined mechanisms of increased precursor supply, the creation of a metabolic sink and the induction of endogenous gene expression in the committed part of the carotenoid pathway. However, AtDXS and AtOR affected overlapping sets of endogenous carotenogenic genes through different mechanisms. AtDXS induced the expression of OsPDS and OsLYCE, whereas AtOR induced all the endogenous carotenogenic genes we tested, with the exception of OsPDS. These experiments offer insight into the bottlenecks in the carotenoid pathway in rice endosperm and will allow the development of more targeted strategies for the creation of engineered plants with particular carotenoid profiles.

**Experimental procedures**

**Plant material**

Wild-type rice (Oryza sativa L. cv. EY110S) and transgenic rice plants were grown in the greenhouse and growth chamber at 28±2°C day/night temperature with a 10-h photoperiod and 60–90% relative humidity for the first 50 days, followed by maintenance at 21±18°C day/night temperature with a 16-h photoperiod thereafter.

**Gene cloning and vector construction**

The AtDXS and AtOR cDNAs were cloned directly from A. thaliana leaf mRNA by RT-PCR using primers designed according to sequences in GenBank (GenBank accession number: NM 203246 and U27099.1). The cDNAs were transferred to vector pGEM-T Easy (Promega, Madison, WI, USA), and the recombinant vectors were digested with EcoRI to release the cDNAs. AtDXS was inserted into vector pPSP (Su et al., 2001) containing the endosperm-specific rice prolamin promoter and the ADPGPP terminator, and AtOR was inserted into vector p326 (Stoger et al., 1999) containing the endosperm-specific wheat low-molecular-weight glutenin gene promoter and nos terminator. Both vectors were linearized with EcoRI to accept the inserts.

The maize PSY1 cDNA was cloned from maize inbred line B73 endosperm by RT-PCR using forward primer 5'-AGG ATC CAT GCC CAT CAT ACT CGT AGG AG-3' incorporating a BamHI site (underlined) and reverse primer 5'-AGA ATT GTA GGT GTG CCG ATT TCT CAA TG-3' incorporating an EcoRI site (underlined). The primers were designed based on sequences in GenBank (GenBank accession number: AY324431). The product was transferred to vector pGEM-T Easy (Promega) to generate pGEM-ZmPSY1 for sequencing and then inserted into vector p326 as above (Stoger et al., 1999).

The Pantoea ananatis (formerly Erwinia uredovora) phytoene desaturase gene (CRTI) was fused in frame with the transit peptide signal from the Phaseolus vulgaris small subunit of ribulose bisphosphate carboxylase (Schreier et al., 1985) in plasmid pPYET4 (Misawa et al., 1994) and amplified by PCR using forward primer 5'-ATC TAG AAT GGC TTC TAG ATC TTC TTC-3' incorporating an XbaI site (underlined) and reverse primer 5'-AGA ATT GTA AAG TAC GAT CCT CTC TCT-3' incorporating an EcoRI site (underlined). The primers were designed based on sequences in GenBank (GenBank accession number: D90087). The product was transferred to vector pGEM-T Easy to produce pGEM-PaCRTI for sequencing and then inserted into pHorP-P containing the endosperm-specific barley D-hordein promoter and the rice ADPGPP terminator (Sorensen et al., 1996).

**Transformation of rice plants**

Seven-day-old mature zygotic rice embryos were transferred to MS osmoticum medium (4.4 g/L Murashige-Skoog salts supplemented with 0.3 g/L casein hydrolysate, 0.5 g/L proline, 72.8 g/L mannitol and 30 g/L sucrose) 4 h before transformation and then bombarded with 10 mg gold particles coated with the carotenoid clearance dioxygenase 1 was inversely correlated with the accumulation of carotenoids in maize endosperm (da Silva Messias et al., 2014; Vallabhaneni et al., 2010). The activities of various carotenoid cleavage dioxygenases might be different in Kaybonnet in the case of Golden Rice II and EY110S in our experiments. Our results clearly demonstrate a mechanistic basis for differences in carotenoid profiles between plants with different transgenic complements and genetic backgrounds, and also significant differences between the differentiated endosperm of intact plants and the in vitro screening system based on rice callus (Bai et al., 2014).

**mRNA blot analysis**

Total RNA was extracted from rice endosperm 25 dap. We separated 25 µg denatured total RNA by 1.2% (w/v) agarose-formaldehyde gel electrophoresis in 1× MOPS buffer and transferred the fractionated RNA to a membrane by capillary blotting (Sambrook et al., 1989). The membrane was probed with digoxigenin-labelled partial cDNAs prepared using the

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Quantitative real-time PCR

Real-time PCR was used to amplify RNA isolated from rice endosperm (25 dap) on a Bio-Rad CFX96TM system using a 25-μl mixture containing 10 ng of synthesized cDNA, 1 x iQ SYBR green supermix (Bio-Rad, Hercules, CA) and 0.2 μM forward and reverse primers for the target genes. To calculate relative expression levels, serial dilutions (0.2–125 ng) were used to produce standard curves for each gene. PCRs were carried out in triplicate using 96-well optical reaction plates, comprising a heating step for 3 min at 95°C, followed by 40 cycles of 95°C for 15 s, 58.5°C for 1 min and 72°C for 20 s. Amplification specificity was confirmed by melt curve analysis on the final PCR products in the temperature range 50–90°C with fluorescence acquired after each 0.5°C increment. The fluorescence threshold value and gene expression data were calculated using the CFX96 system software Bio-Rad, Hercules, CA. Values represent the mean of three real-time PCR replicates ±SD. The forward and reverse primers for each transgene are shown in Table S1.

Transmission electron microscopy

Rice seed sections (0.5 × 2.0 mm) were fixed in 2.5% v/v glutaraldehyde and 2.0% v/v paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4°C. The sections were washed three times in 0.1 M sodium phosphate buffer (pH 7.2), and post-fixed in 1% v/v osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2) for 1 h. They were then washed three times in re-distilled water and dehydrated in an alcohol series (30–100%) before embedding in epoxy resin Araldite® Embed 812 (Epon-812) from Anane Electron Microscopy Sciences, Madrid, Spain (URL: www.aname.es) and polymerizing at 60°C. Ultra-thin sections (80–90 nm) were prepared with a diamond knife using a Reichter Jung Ultramicrotome Ultracut E (Scotland), mounted on SPI-ChemTM Formvar/carbon-coated copper grids, and stained with uranyl acetate and Reynolds’s lead citrate prior to examination using an EM 910 transmission electron microscope (Carl Zeiss, Jena, Germany).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Carotenoid profiles of transgenic endosperm determined by HPLC. HPLC analysis shows the profile of carotenoids in the T3 endosperm (40 DAP) of transgenic rice lines L, D and O (mature seeds). Line names are defined in the legend to Figure 3. No carotenoids detected in wild type of rice endosperm.

Table S1 Oligonucleotide sequences of forward (F) and reverse (R) primers for mRNA blot analysis.

Table S2 Oligonucleotide sequences of forward (F) and reverse (R) primers for quantitative real-time PCR analysis.