Plant Biotechnology Journal

Plant Biotechnology Journal (2016) **14**, pp. 195–205 doi: 10.1111/pbi.12373

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

Chao Bai[1], Teresa Capell[1], Judit Berman[1], Vicente Medina[1], Gerhard Sandmann[2], Paul Christou[1,3] and Changfu Zhu[1,*]

[1]*Department of Plant Production and Forestry Science, ETSEA, University of Lleida-Agrotecnio Center, Lleida, Spain*
[2]*Institute of Molecular Bioscience, J. W. Goethe University, Frankfurt am Main, Germany*
[3]*Institucio Catalana de Recerca i Estudis Avancats, Passeig Lluís Companys, Barcelona, Spain*

Received 6 December 2014;
revised 23 February 2015;
accepted 2 March 2015
**Correspondence* (Tel +34 973702694; fax 0034-973-702515; email zhu@pvcf.udl.cat)

Keywords: rice (*Oryza sativa*), carotenoids, secondary metabolites, 1-deoxy-D-xylulose 5-phosphate synthase, *ORANGE* gene, multigene engineering.

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; Von Lintig 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 RP5 prolamin, wheat low-molecular-weight glutenin and barley D-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).

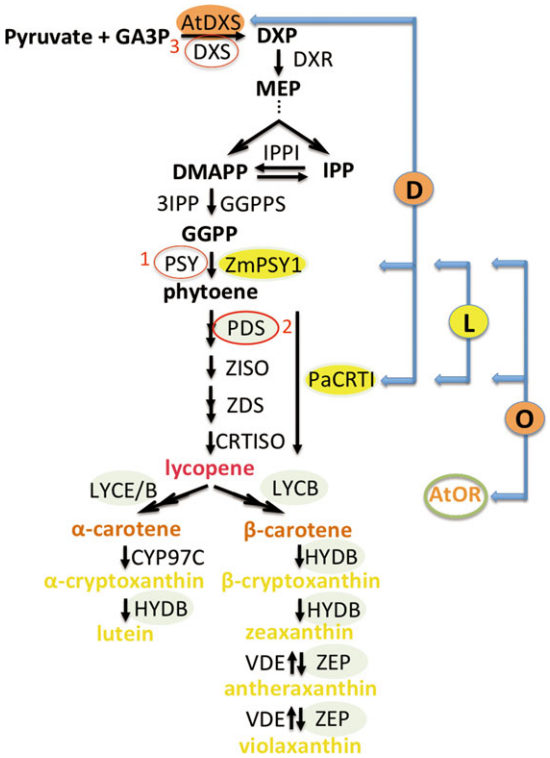

Figure 1 Reconstruction of the carotenoid biosynthesis pathway in rice endosperm (Bai *et al.*, 2011; Farre *et al.*, 2010). GA3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; AtDXS, Arabidopsis DXS; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; 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, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β-cyclase; LYCE, lycopene ε-cyclase; CYP97C, carotene ε-ring hydroxylase; HYDB, 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.

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

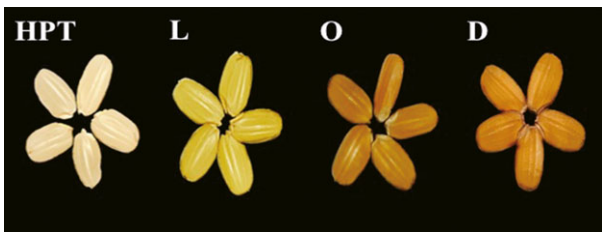

Figure 2 Phenotype of transgenic rice seeds expressing different combinations of carotenogenic genes. Two different endosperm phenotypes were observed. The orange seed endosperm co-expressed *AtDXS*, *ZmPSY1* and *PaCRTI* (genotype D), or *AtOR*, *ZmPSY1* and *PaCRTI* (genotype O), whereas the yellow seed endosperm (genotype L) co-expressed *ZmPSY1* and *PaCRTI.* Abbreviations: HPT, seed endosperm expressing *hpt*; L, seed endosperm expressing *ZmPSY1* and *PaCRTI*; O, seed endosperm expressing *ZmPSY1, PaCRTI* and *AtOR*; D, seed endosperm expressing *ZmPSY1, PaCRTI* and *AtDXS*.

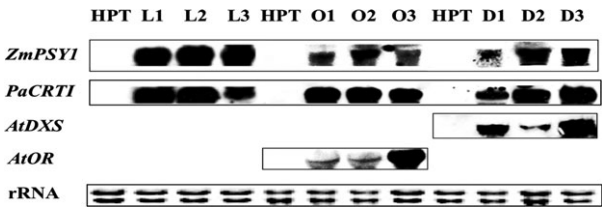

Figure 3 Transgenic expression analyses in rice endosperm. Transgenic expression in T3 rice endosperm at 25 dap (25 μg total RNA per sample). Abbreviations: HPT, seed endosperm expressing *hpt*; L1, L2 and L3, seed endosperm expressing *ZmPSY1* and *PaCRTI*; O1, O2 and O3, seed endosperm expressing *ZmPSY1, PaCRTI* and *AtOR*; D1, D2 and D3, seed endosperm expressing *ZmPSY1, PaCRTI* and *AtDXS*.

25.83 μg/g DW, respectively (Table 1). The carotenoid levels in the orange endosperm of genotype O were therefore 2.1- to 4.7-fold higher than the yellow endosperm of genotype L. Similarly, the carotenoid levels in the orange endosperm of genotype D were 2.7- to 5.8-fold higher than genotype L (Figure 4). The proportion of total carotenoids represented by β-carotene was 25–39% in genotype L, 47–52% in genotype D and 40–50% in genotype O, representing average yields of 1.6, 8.3 and 10.8 μg/g DW, respectively (Table 1, Figure S1). The levels of α-carotene were also higher in genotypes D and O compared to L, with averages of 6.1, 6.0 and 1.2, μg/g DW, respectively. Lutein levels were much higher in genotype O (average 1.0 μg/g DW) than genotypes D (average 0.4 μg/g DW) or L (average 0.2 μg/g DW) (Table 1, Figure S1).

AtOR and *AtDXS* up-regulate different sets of endogenous carotenogenic genes

The expression of the *ZmPSY1*, *PaCRTI*, *AtDXS* and *AtOR* genes in the endosperm of transgenic rice plants was analysed by mRNA blot at 25 days after pollination (dap) as shown in Figure 3. As anticipated, *ZmPSY1* and *PaCRTI* mRNA accumulated in all three genotypes but not in the *hpt*-only control, *AtDXS* mRNA was detected solely in genotype D, and *AtOR* mRNA was detected solely in genotype O (Figure 3).

We also monitored the expression of endogenous carotenogenic genes to determine whether they were regulated in response to transgene expression. We measured the levels of the

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

	L1	L2	L3	O1	O2	O3	D1	D2	D3
Violaxanthin	0	0	0	0.12 ± 0	0	0.25 ± 0.03	0	0	0.06 ± 0.01
Antheraxanthin		0	0	0.08 ± 0.05	0	0	0	0	0
Zeaxanthin	0.03 ± 0.03	0.09 ± 0.02	0.17 ± 0.13	0.23 ± 0.14	0.29 ± 0.22	0.57 ± 0.18	0.22 ± 0.15	0.13 ± 0.22	0.33 ± 0.12
β-Cryptoxanthin	0.17 ± 0.30	0	0.15 ± 0.27	0.19 ± 0.33	0.40 ± 0.70	1.13 ± 0.03	0.41 ± 0.72	0.39 ± 0.67	0.41 ± 0.72
β-Carotene	1.48 ± 0.02	2.15 ± 0.09	1.17 ± 0.19	5.87 ± 1.44	8.47 ± 1.23	10.52 ± 1.64	8.36 ± 0.44	7.50 ± 1.25	16.61 ± 1.37
Lutein	0.16 ± 0.09	0.26 ± 0.02	0.26 ± 0.09	0.55 ± 0.07	0.58 ± 0.20	1.73 ± 0.16	0.39 ± 0.20	0.26 ± 0.16	0.69 ± 0.39
α-Cryptoxanthin	0.53 ± 0.01	0.61 ± 0.04	0.42 ± 0.08	0.82 ± 0.13	1.49 ± 0.10	1.63 ± 0.16	1.74 ± 0.08	1.48 ± 0.10	2.09 ± 0.68
α-Carotene	0.90 ± 0.10	1.90 ± 0.00	0.70 ± 0.10	3.20 ± 0.50	5.00 ± 1.00	9.70 ± 0.80	5.00 ± 0.10	3.60 ± 0.70	9.70 ± 1.60
Phytoene	2.16 ± 0.05	0.50 ± 0.01	1.74 ± 0.06	0.47 ± 0.06	2.36 ± 0.35	0.35 ± 0.30	1.67 ± 0.52	1.58 ± 0.12	1.89 ± 0.28
Total Carotenoids	5.43 ± 0.37	5.51 ± 0.16	4.61 ± 0.81	11.53 ± 2.33	18.59 ± 1.64	25.83 ± 2.37	17.79 ± 1.12	14.94 ± 1.12	31.78 ± 5.01
β/α	1.06	0.81	1.08	1.42	1.30	0.95	1.26	1.50	1.40
% β-Carotene	27.26	39.02	25.38	50.91	45.56	40.73	46.99	50.20	52.27

Data are means ± SD from analysis of three independent seed batches (40 dap) and are expressed as μg/g DW. β/α, the ratio of β,β-carotenoids to β,ε-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 *AtDXS*; L, seed endosperm expressing *ZmPSY1* and *PaCRTI*.

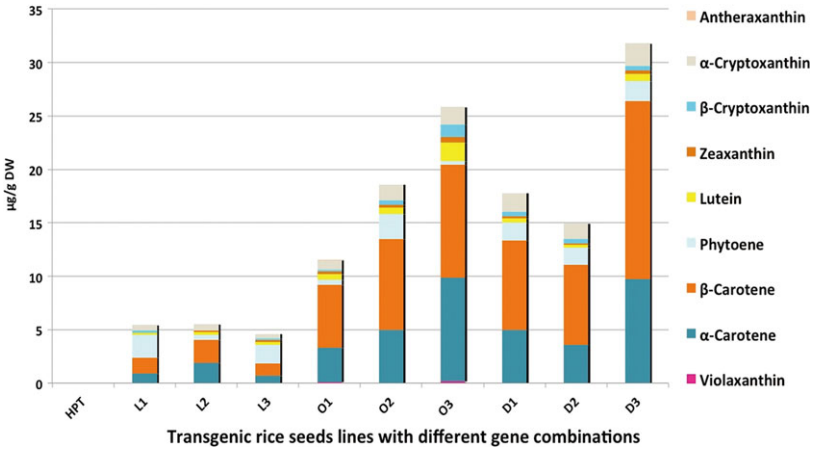

Figure 4 Carotenoid content and composition of transgenic rice endosperm. The carotenoid content and composition of all lines were analysed by UHPLC. Bars represent different carotenoids in lines expressing different transgenic combinations. All carotenoid content data were averaged for three independent measurements (T3 mature seeds) at 40 dap. Column names are defined in the legend to Figure 3.

endogenous phytoene desaturase (*OsPDS*), lycopene ε-cyclase (*OsLYCE*), lycopene β-cyclase (*OsLYCB*), β-carotene hydroxylase (*OsBCH2*) 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. *OsBCH2* expression was suppressed in lines D1 and D2 but not in line D3, 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 products (Burkhardt *et al.*, 1997). Similarly, the expression of bacterial CRTI (which replaces several consecutive desaturation and isomerization steps catalyzed by the plant enzymes PDS, Z-ISO, ZDS and CRTISO) alone was insufficient to relieve the bottleneck and no carotenoids accumulated in the endosperm due to the absence of PSY activity (Chao Bai, Changfu Zhu, Teresa Capell, Paul Christou.). Therefore, carotenoid accumulation in rice requires at least two enzymes: PSY and CRTI. This approach was demonstrated during the development of Golden Rice, which expressed daffodil PSY and PaCRTI to produce 1.6 μg/g DW of carotenoids in the endosperm (Ye *et al.*, 2000). The yield was later improved to 37 μg/g DW by replacing the daffodil enzyme with the more active ZmPSY1 (Paine *et al.*, 2005).

Both the Golden Rice and Golden Rice II lines accumulate predominantly β-carotene at the expense of other carotenoid intermediates earlier in the pathway, and they are notable for the almost complete absence of phytoene, the immediate 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*.

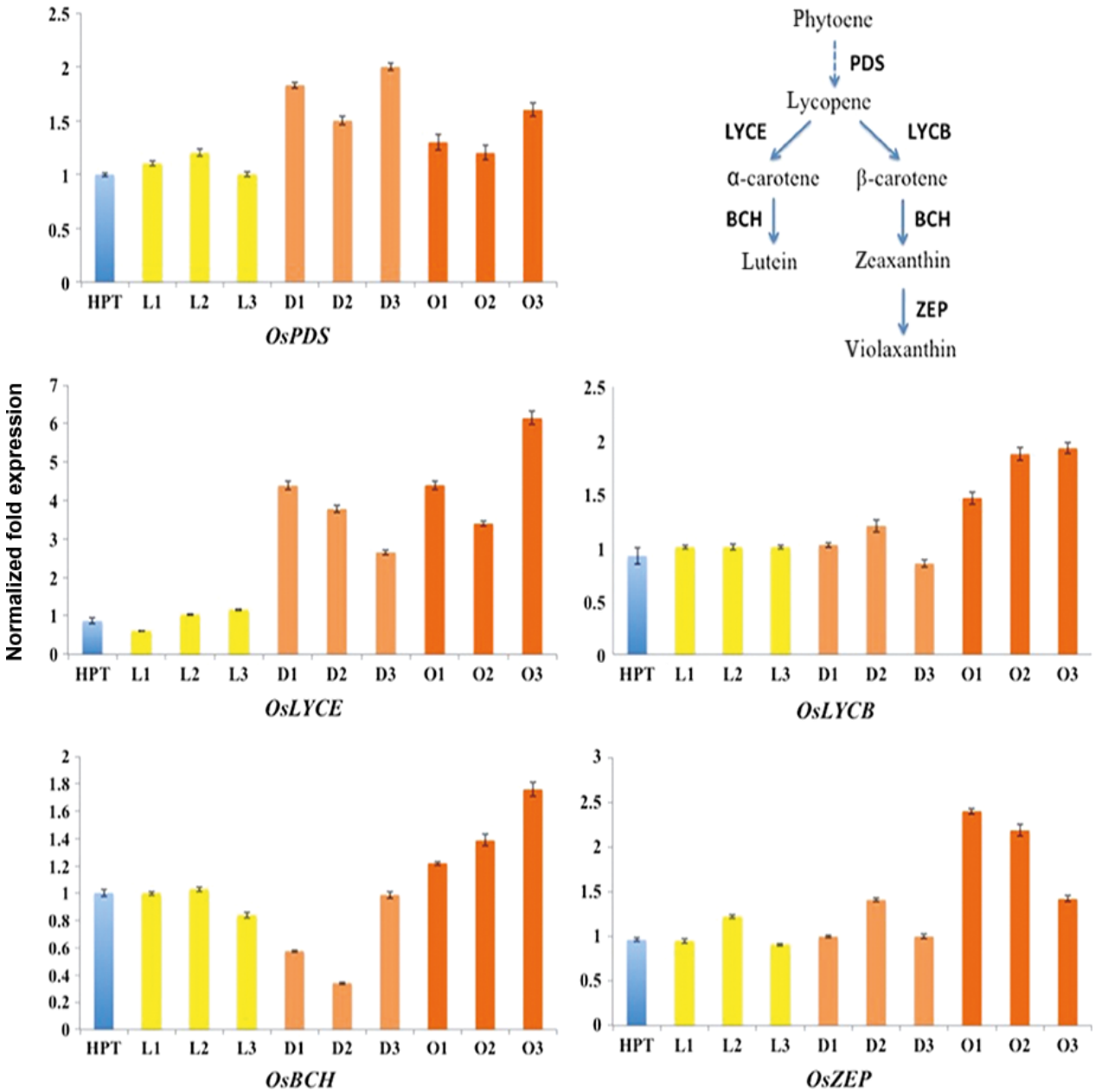

Figure 5 Expression of endogenous carotenogenic genes in transgenic rice endosperm. Relative transcript levels of endogenous *OsPDS, OsLYCE*, *OsLYCB*, *OsBCH2* and *OsZEP* genes in T3 rice endosperm at 25 dap expressing different transgenic combinations. Values are means ± SD of three quantitative real-time PCR replicates. The expression for each gene was normalized against *actin* mRNA. Abbreviations: *PDS*, phytoene desaturase; *LYCE*, lycopene ε-cyclase; *LYCB*, lycopene β-cyclase; *BCH*, β-carotene hydroxylase; *ZEP*, zeaxanthin epoxidase. Line names are defined in the legend to Figure 3.

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 β-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

endosperm in genotype D was orange compared to the yellow endosperm of 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).

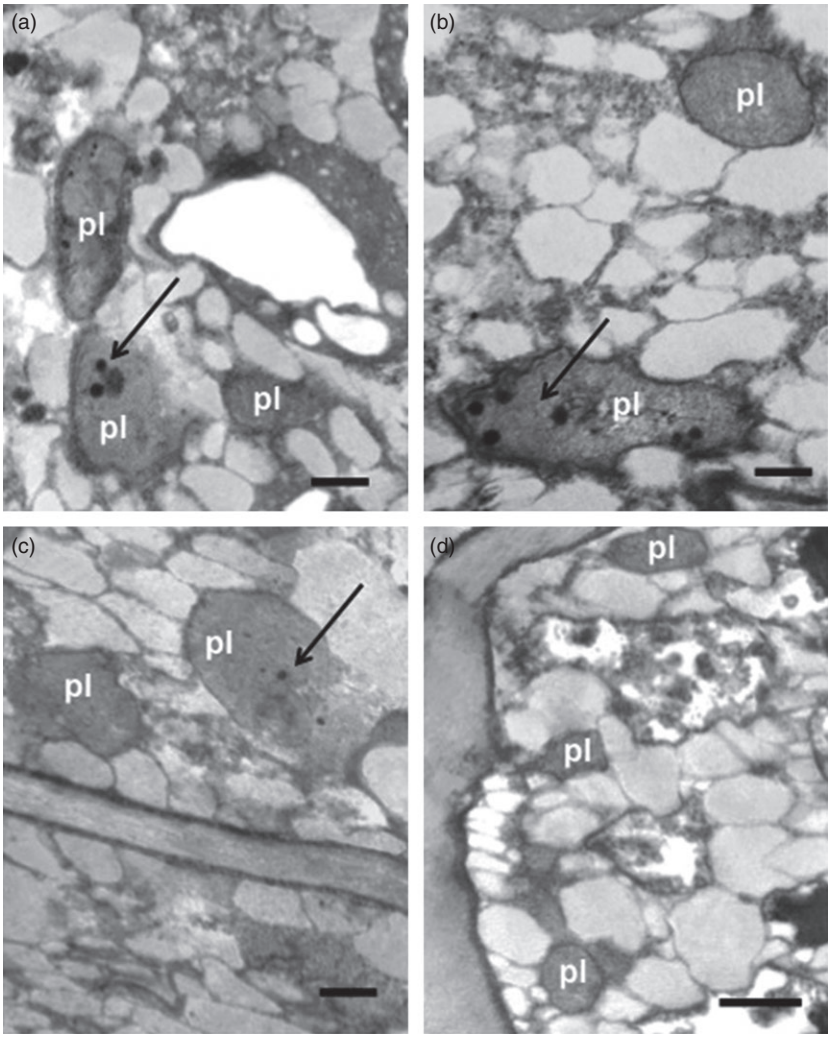

Figure 6 Transmission electron micrographs of transgenic rice endosperm expressing *ZmPSY1*, *PaCRTI* and *AtOR* (a), *ZmPSY1*, *PaCRTI* and *AtDXS* (b), *ZmPSY1* and *PaCRTI* (c), *HPT* (d). Arrows indicate plastoglobuli inside plastids (pl). Scale bar 0.3 μm (a, b and c) and 1 μm (d).

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; Ye *et al.*, 2000). 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 EYI105 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

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 (Naqvi *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 EYI105 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. EYI105) and transgenic rice plants were grown in the greenhouse and growth chamber at 28/20 °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 pRP5 (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 GGC CAT CAT ACT CGT ACG AG-3′ incorporating a BamHI site (underlined) and reverse primer 5′-AGA ATT CTA GGT CTG GCC 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 pYPIET4 (Misawa *et al.*, 1994) and amplified by PCR using forward primer 5′-ATC TAG AAT GGC TTC TAT GAT ATC CTC TTC-3′ incorporating an XbaI site (underlined) and reverse primer 5′-AGA ATT CTC AAA TCA GAT CCT CCA GCA TCA-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 carotenogenic constructs and the selectable maker *hpt* at a 3 : 3 : 1 or 3 : 3 : 3 : 1 ratio as appropriate (Christou, 1997; Christou *et al.*, 1991). The embryos were returned to osmoticum medium for 12 h before selection on MS medium (as above but without mannitol) supplemented with 30 mg/L hygromycin and 2.5 mg/L 2,4-dichlorophenoxyacetic acid in the dark for 2–3 weeks (Farré *et al.*, 2012). Transgenic plantlets were regenerated and hardened off in soil.

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

PCR-DIG Probe Synthesis Kit (Roche, Mannheim, Germany), with hybridization carried out at 50 °C overnight using DIG Easy Hyb (Roche). The membrane was washed twice for 5 min in 2× SSC, 0.1% SDS at room temperature, twice for 20 min in 0.2× SSC, 0.1% SDS at 68 °C and then twice for 10 min in 0.1× SSC, 0.1% SDS at 68 °C. After immunological detection with anti-DIG-AP (Fab-Fragments Diagnostics GmbH, Roche, Mannheim, Germany), chemiluminescence generated by disodium [3-(1-chloro-3′-methoxy-spiro[adamantane-4,4′-dioxetane]-3′-yl)phenyl] phosphate (CSPD) (Roche) was detected on Kodak BioMax light film (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. The forward and reverse primers for each transgene used for probe synthesis are shown in Table S1.

Carotenoid extraction and quantification

Carotenoids were extracted in darkness from 50 mg freeze-dried endosperm (40 dap) with 50/50 (vol/vol) tetrahydrofuran and methanol at 60 °C for 20 min. The mixture was filtered and the residue re-extracted in acetone. Chromatography was carried out using a Waters ACQUITY UPLC™ system (Waters, Milford, MA) comprising an ACQUITY UPLC™ binary solvent manager and an ACQUITY UPLC™ sample manager, coupled to a photodiode array (PDA) 2996 detector. This was linked to an Acquity™ TQD tandem-quadrupole MS equipped with a Z-spray electrospray interface (Manchester, UK). MassLynx™ software version 4.1 (Waters) was used for data acquisition and processing. Compounds were separated with an ACQUITY UPLC™ BEH C18 column (1.7 μm; 1 × 150 mm) (Waters) and a gradient system with the mobile phase consisting of solvent A (7 : 3 v/v acetone : methanol) and solvent B (100% water). The linear gradient was as follows: 0–1.0 min, 25% B, 0.5 mL/min (isocratic); 1.0–10.0 min, 4.9% B 0.5 mL/min (linear gradient); 10.0–11.4 min, 0% B, 0.7 mL/min (linear gradient); 11.4–19.2 min, 0% B, 0.7 mL/min (isocratic); 19.2–20.0 min, 25% B, 0.5 mL/min (linear gradient); and 20.0–22.0 min, 25% B, 0.5 mL/min (isocratic). The injection needle was washed with acetone : methanol (7 : 3 v/v) and subsequently with 2-propanol between injections. The injection volume was 5 μL and the column was kept at 32 °C while the temperature in the sample manager was maintained at 25 °C. The average maximum pressure in the chromatographic system was 15 000 psi (Delpino-Rius *et al.*, 2014).

Carotenoids were identified using reference compounds as standards and by their spectral properties. Lutein, β-cryptoxanthin and β-carotene were purchased from Sigma (St Louis, MO); zeaxanthin from Fluka (Buchs, Switzerland); and phytoene, α-carotene, violaxanthin and antheraxanthin from CaroteNature (Lupsingen, Switzerland). The hydroxylated carotenoids were further characterized by mass spectroscopy. Optical absorbance maxima and masses were for α-cryptoxanthin 420, 445, 475 nm with m/z 552; for β-cryptoxanthin 425, 450, 475 nm with m/z 552; for lutein 422, 445, 475 nm with m/z 568; and for zeaxanthin 425, 450, 476 nm with m/z 568. Quantification was carried out with calibration curves of the standards. In the case of α-cyptoxanthin, the calibration curve for α-carotene was used as the latter carotenoid exhibits very similar absorbance properties. Total carotenoid content in extracts was calculated spectrophotometrically using the absorbance at 450 nm and the average extinction coefficient $A^{1\%}_{1cm}$ of 2332 as lutein and zeaxanthin represented the majority of the carotenoids.

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 × 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 S2.

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% w/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 Aname 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 (Scotia), mounted on SPI-ChemTM Formvar/carbon-coated copper grids, and stained with uranyl acetate and Reynold's lead citrate prior to examination using an EM 910 transmission electron microscope (Carl Zeiss, Jena, Germany).

Acknowledgements

This study was supported by the Ministerio de Economia y Competitividad, Spain (BIO2011-22525 and PIM2010PKB-00746 CAROMAIZE), European Research Council Advanced Grant (BIOFORCE) to PC. C.B. is the recipient of a PhD fellowship from the Universitat de Lleida, Spain.

References

Auldridge, M.E., Block, A., Vogel, J.T., Dabney-Smith, C., Mila, I., Bouzayen, M., Magallanes-Lundback, M., DellaPenna, D., McCarty, D.R. and Klee, H.J. (2006) Characterization of three members of the Arabidopsis carotenoid cleavage dioxygenase family demonstrates the divergent roles of this multifunctional enzyme family. *Plant J.* **45**, 982–993.

Bai, C., Twyman, R.M., Farré, G., Sanahuja, G., Christou, P., Capell, T. and Zhu, C. (2011) A golden era-pro-vitamin A enhancement in diverse crops. *In Vitro Cell. Dev. Biol. Plant*, **47**, 205–221.

Bai, C., Rivera, S.M., Medina, V., Alves, R., Vilaprinyo, E., Sorribas, A., Canela, R., Capell, T., Sandmann, G., Christou, P. and Zhu, C. (2014) An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation. *Plant J.* **77**, 464–475.

Burkhardt, P.K., Beyer, P., Wunn, J., Kloti, A., Armstrong, G.A., Schledz, M., von, L.J. and Potrykus, I. (1997) Transgenic rice (*Oryza sativa*) endosperm

expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis. *Plant J.* **11**, 1071–1078.

Campbell, R., Ducreux, L.J., Morris, W.L., Morris, J.A., Suttle, J.C., Ramsay, G., Bryan, G.J., Hedley, P.E. and Taylor, M.A. (2010) The metabolic and developmental roles of carotenoid cleavage dioxygenase 4 form potato. *Plant Physiol.* **154**, 656–664.

Cazzonelli, C.I. and Pogson, B.J. (2010) Source to sink: regulation of carotenoids biosynthesis in plants. *Trends Plant Sci.* **15**, 266–274.

Christou, P. (1997) Rice transformation by bombardment. *Plant Mol. Biol.* **35**, 197–203.

Christou, P., Ford, T. and Kofron, M. (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Nat. Biotechnol.* **9**, 957–962.

Delpino-Rius, A., Eras, J., Marsol-Vall, A., Vilaró, F., Balcells, M. and Canela-Garayoa, R. (2014) Ultra performance liquid chromatography analysis to study the changes in the carotenoid profile of commercial monovarietal fruit juices. *J. Chromatogr. A*, **1331**, 90–99.

Eisenreich, W., Rohdich, F. and Bacher, A. (2001) Deoxyxylulose phosphate pathway to terpenoids. *Trends Plant Sci.* **6**, 78–84.

Enfissi, E.M.A., Fraser, P.D., Lois, L.M., Boronat, A., Schuch, W. and Bramley, P. (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotechnol. J.* **3**, 17–27.

Estévez, J.M., Cantero, A., Reindl, A., Reichler, S. and Leon, P. (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *J. Biol. Chem.* **276**, 22901–22909.

Farre, G., Sanahuja, G., Naqvi, S., Bai, C., Capell, T., Zhu, C.F. and Christou, P. (2010) Travel advice on the road to carotenoids in plants. *Plant Sci.* **179**, 28–48.

Farré, G., Naqvi, S., Sanahuja, G., Bai, C., Zorrilla-López, U., Rivera, S.M., Canela, R., Sandman, G., Twyman, R.M., Capell, T., Zhu, C. and Christou, P. (2012) Combinatorial genetic transformation of cereals and the creation of metabolic libraries for the carotenoid pathway. *Methods Mol. Biol.* **847**, 419–435.

Fraser, P.D. and Bramley, P.M. (2004) The biosynthesis and nutritional uses of carotenoids. *Prog. Lipid Res.* **43**, 228–265.

Fraser, P.D., Enfissi, E.M.A., Halket, J.M., Truesdale, M.R., Yu, D., Gerrish, C. and Bramley, P.M. (2007) Manipulation of phytoene levels in tomato fruit: effects on isoprenoids, plastids, and intermediary metabolism. *Plant Cell*, **19**, 3194–3211.

Giuliano, G. and Diretto, G. (2007) Of chromoplasts and chaperones. *Trends Plant Sci.* **12**, 529–531.

Harjes, C.E., Rocheford, T.R., Bai, L., Brutnell, T.P., Kandianis, C.B., Sowinski, S.G., Stapleton, A.E., Vallabhaneni, R., Williams, M., Wurtzel, E.T., Yan, J. and Buckler, E.S. (2008) Natural genetic variation in *Lycopene Epsilon Cyclase* tapped for maize biofortification. *Science*, **319**, 330–333.

Kean, E.G., Ejeta, G., Hamaker, B. and Ferruzzi, M.G. (2007) Characterization of carotenoid pigments in mature and developing kernels of select yellow-endosperm sorghum varieties. *J. Agric. Food Chem.* **55**, 2619–2626.

Kim, S.H., Ahn, Y.O., Ahn, M.J., Jeong, J.C., Lee, H.S. and Kwak, S.S. (2013) Cloning and characterization of an *Orange* gene that increases carotenoid accumulation and salt stress tolerance in transgenic sweetpotato cultures. *Plant Physiol. Biochem.* **70**, 445–454.

Li, L. and Van Eck, J. (2007) Metabolic engineering of carotenoids accumulation by creating a metabolic sink. *Transgenic Res.* **16**, 581–585.

Li, L., Paolillo, D.J., Parthasarathy, M.V., DiMuzio, E.M. and Garvin, D.F. (2001) A novel gene mutation that confers abnormal patterns of beta-carotene accumulation in cauliflower (*Brassica oleracea* var. *botrytis*). *Plant J.* **26**, 59–67.

Li, L., Lu, S., Cosman, K.M., Earle, E.D., Garvin, D.F. and O'Neill, J. (2006) β-carotene accumulation induced by the cauliflower *Or* gene is not due to an increased capacity of biosynthesis. *Phytochemistry*, **67**, 1177–1184.

Lopez, A.B., Van Eck, J., Conlin, B.J., Paolillo, D.J., O'Neill, J. and Li, L. (2008) Effect of the cauliflower *Or* transgene on carotenoids accumulation and chromoplast formation in transgenic potato tubers. *J. Exp. Bot.* **59**, 213–223.

Lu, S. and Li, L. (2008) Carotenoid metabolism: biosynthesis, regulation, and beyond. *J. Integr. Plant Biol.* **50**, 778–785.

Lu, S., Van Eck, J., Zhou, X.J., Lopez, A.B., O'Halloran, D.M., Cosman, K.M., Conlin, B.J., Paolillo, D.J., Garvin, D.F., Vrebalov, J., Kochian, L.V., Kupper, H., Earle, E.D., Cao, J. and Li, L. (2006) The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high-levels of β-carotene accumulation. *Plant Cell*, **18**, 3594–3605.

Maass, D., Arango, J., Wust, F., Beyer, P. and Welsch, R. (2009) Carotenoid crystal formation in *Arabidopsis* and carrot roots caused by increased phytoene synthase protein levels. *PLoS ONE*, **4**, e6373.

Miralpeix, B., Rischer, H., Häkkinen, S.T., Ritala, A., Seppänen-Laakso, T., Oksman-Caldentey, K.M., Capell, T. and Christou, P. (2013) Metabolic engineering of plant secondary products: which way forward? *Curr. Pharm. Des.* **19**, 5622–5639.

Misawa, N., Yamano, S., Linden, H., de Felipe, M.R., Lucas, M., Ikenaga, H. and Sandmann, G. (1994) Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crtI* in transgenic plants showing an increase of β-carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon. *Plant J.* **4**, 833–840.

Morris, W.L., Ducreux, L.J.M., Hedden, P., Millam, S. and Taylor, M.A. (2006) Overexpression of a bacterial 1-deoxy-D-xylulose 5-phosphate synthase gene in potato tubers perturbs the isoprenoid metabolic network: implications for the control of the tuber life cycle. *J. Exp. Bot.* **57**, 3007–3018.

Nisar, N., Li, L., Lu, S., Khin, N.C. and Pogson, B.J. (2015) Carotenoid metabolism in plants. *Mol Plant.* **8**, 68–82.

Nogueira, M., Mora, L., Enfissi, E.M., Bramley, P.M. and Fraser, P.D. (2013) Subchromoplast sequestration of carotenoids affects regulatory mechanisms in tomato lines expressing different carotenoid gene combinations. *Plant Cell*, **25**, 4560–4579.

O'Connor, S.E. and Maresh, J.J. (2006) Chemistry and biology of monoterpene indole alkaloid biosynthesis. *Nat. Prod. Rep.* **23**, 532–547.

Ohmiya, A., Kishimoto, S., Aida, R., Yoshioka, S. and Sumitomo, K. (2006) Carotenoid cleavage dioxygenase (CmCCD4a) contributes to white color formation in Chrysanthemum Petals. *Plant Physiol.* **142**, 1193–1201.

Paine, J.A., Shipton, C.A., Chaggar, S., Howells, R.M., Kennedy, M.J., Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L. and Drake, R. (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotechnol.* **23**, 482–487.

Rischer, H., Häkkinen, S.T., Ritala, A., Seppänen-Laakso, T., Miralpeix, B., Capell, T., Christou, P. and Oksman-Caldentey, K.M. (2013) Plant cells as pharmaceutical factories. *Curr. Pharm. Des.* **19**, 5640–5660.

Rodríguez-Concepción, M. (2010) Supply of precursors for carotenoid biosynthesis in plants. *Arch. Biochem. Biophys.* **504**, 118–122.

Rodríguez-Concepción, M. and Boronat, A. (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. *Plant Physiol.* **130**, 1079–1089.

Sambrook, J., Fritschi, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, **1**, 63–70.

Schaub, P., Al-Babili, S., Drake, R. and Beyer, P. (2005) Why is golden rice golden (yellow) instead of red? *Plant Physiol.* **138**, 441–450.

Schreier, P., Seftor, E., Schell, J. and Bohnert, H. (1985) The use of nuclear-encoded sequences to direct the light regulated synthesis and transport of a foreign protein into plant chloroplasts. *EMBO J.* **4**, 25–32.

da Silva Messias, R., Galli, V., Dos Anjos, E., Silva, S.D. and Rombaldi, C.V. (2014) Carotenoid biosynthetic and catabolic pathways: gene expression and carotenoid content in grains of maize landraces. *Nutrients*, **6**, 546–563.

Sorensen, M.B., Muller, M., Skerritt, J. and Simpson, D. (1996) Hordein promoter methylation and transcriptional activity in wild-type and mutant barley endosperm. *Mol. Gen. Genet.* **250**, 750–760.

Stoger, E., Williams, S., Keen, D. and Christou, P. (1999) Constitutive versus seed specific expression in transgenic wheat: temporal and spatial control. *Transgenic Res.* **8**, 73–82.

Su, P.H., Yu, S.M. and Chen, C.S. (2001) Spatial and temporal expression of a rice prolamin gene RP5 promoter in transgenic tobacco and rice. *J. Plant Physiol.* **158**, 247–254.

Underwood, B.A. and Arthur, P. (1996) The contribution of vitamin A to public health. *FASEB J.* **10**, 1040–1048.

Vallabhaneni, R., Bradbury, L.M. and Wurtzel, E.T. (2010) The carotenoid dioxygenase gene family in maize, sorghum, and rice. *Arch. Biochem. Biophys.* **504**, 104–111.

Von Lintig, J. and Vogt, K. (2004) Vitamin A formation in animals: molecular identification and functional characterization of carotene cleaving enzymes. *J. Nutr.* **134**, 251S–256S.

Ye, X., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P. and Potrykus, I. (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoids-free) rice endosperm. *Science*, **287**, 303–305.

Zhu, C., Naqvi, S., Gomez-Galera, S., Pelacho, A.M., Capell, T. and Christou, P. (2007) Transgenic strategies for the nutritional enhancement of plants. *Trends Plant Sci.* **12**, 548–555.

Zhu, C., Naqvi, S., Breitenbach, J., Sandmann, G., Christou, P. and Capell, T. (2008) Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoids pathway in maize. *Proc. Natl Acad. Sci. USA*, **105**, 18232–18237.

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.

