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ORIGINAL ARTICLE

The Arabidopsis *ORANGE* (*AtOR*) gene promotes carotenoid accumulation in transgenic corn hybrids derived from parental lines with limited carotenoid pools

Judit Berman[1] · Uxue Zorrilla-López[1] · Vicente Medina[1] · Gemma Farré[1] · Gerhard Sandmann[2] · Teresa Capell[1] · Paul Christou[1,3] · Changfu Zhu[1]

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Abstract

Key message **The *AtOR* gene enhances carotenoid levels in corn by promoting the formation of plastoglobuli when the carotenoid pool is limited, but has no further effect when carotenoids are already abundant.**

Abstract The cauliflower *orange* (*or*) gene mutation influences carotenoid accumulation in plants by promoting the transition of proplastids into chromoplasts, thus creating intracellular storage compartments that act as metabolic sink. We overexpressed the Arabidopsis *OR* gene under the control of the endosperm-specific wheat LMW glutenin promoter in a white corn variety that normally accumulates only trace amounts of carotenoids. The total endosperm carotenoid content in the best-performing *AtOR* transgenic corn line was 32-fold higher than wild-type controls (~25 µg/g DW at 30 days after pollination) but the principal carotenoids remained the same, suggesting that AtOR increases the abundance of existing carotenoids without changing the metabolic composition. We analyzed the expression of endogenous genes representing the carotenoid biosynthesis and MEP pathways, as well as the plastid fusion/translocation factor required for chromoplast formation, but only the *DXS1* gene was upregulated in the transgenic corn plants. The line expressing *AtOR* at the highest level was crossed with four transgenic corn lines expressing different carotenogenic genes and accumulating different carotenoids. The introgression of *AtOR* increased the carotenoid content of the hybrids when there was a limited carotenoid pool in the parental line, but had no effect when carotenoids were already abundant in the parent. The *AtOR* gene therefore appears to enhance carotenoid levels by promoting the formation of carotenoid-sequestering plastoglobuli when the carotenoid pool is limited, but has no further effect when carotenoids are already abundant because high levels of carotenoids can induce the formation of carotenoid-sequestering plastoglobuli even in the absence of *AtOR*.

Keywords *Orange* gene · Carotenoids · Plastoglobuli · Corn · Transgenic · Hybrids

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✉ Changfu Zhu
zhu@pvcf.udl.cat

[1] Department of Plant Production and Forestry Science, University of Lleida-Agrotecnio Center, Av. Alcalde Rovira Roure, 191, 25198 Lleida, Spain

[2] Biosynthesis Group, Molecular Biosciences, Johann Wolfgang Goethe Universität, 60054 Frankfurt, Germany

[3] ICREA, Catalan Institute for Research and Advanced Studies, Passeig Lluís Companys 23, 08010 Barcelona, Spain

Introduction

Carotenoids are natural pigments synthesized in the plastids of plants and algae, and in some non-photosynthetic organisms such as bacteria and fungi (Zhu et al. 2010). Several different strategies have been used to engineer carotenoid metabolism in plants (Fig. 1). Upstream 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway precursors are rate limiting in, e.g., tomato, potato, and rice (Enfissi et al. 2005; Morris et al. 2006; Bai et al. 2014, 2016), so increasing the precursor supply can direct additional flux into the pathway thus increasing total carotenoid

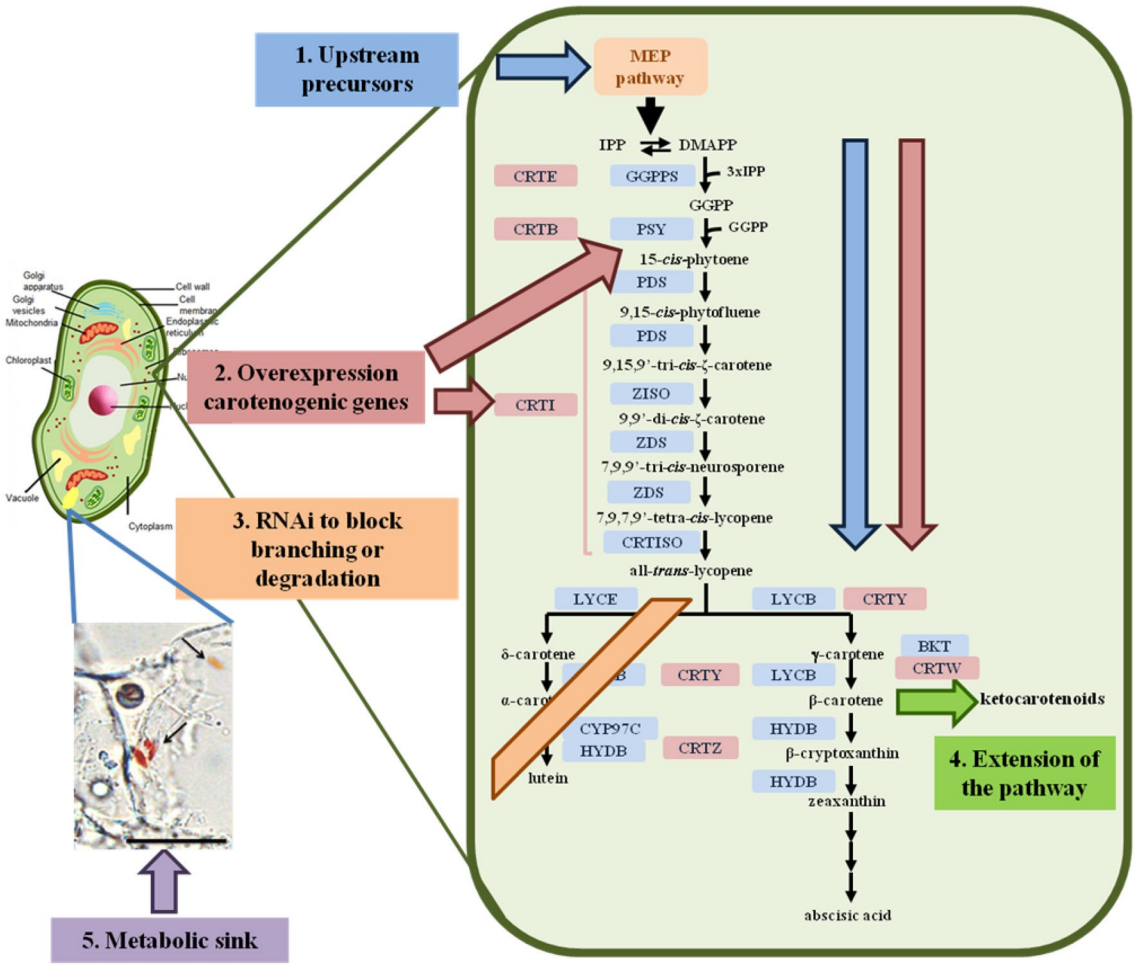

Fig. 1 Strategies to modulate carotenoid levels in plants. IPP isopentenyl diphosphate, DMAPP dimethylallyl diphosphate, GGPP geranylgeranyl diphosphate, GGPPS, GGPP synthase, PSY phytoene synthase, PDS phytoene desaturase, ZISO ζ-carotene isomerase, ZDS ζ-carotene desaturase, CRTISO carotenoid isomerase, LYCB lycopene β-cyclase, LYCE lycopene ε-cyclase, CYP97C carotene ε-ring hydroxylase, HYDB β-carotene hydroxylase, CRTE bacterial GGPP synthase, CRTB bacterial phytoene synthase, CRTI bacterial phytoene desaturase/isomerase, CRTY bacterial lycopene β-cyclase, CRTZ bacterial hydroxylase, BKT β-carotene ketolase, CRTW bacterial β-carotene ketolase (adapted from Berman et al. 2015; light microscopy from Bai et al. 2014; *scale bar* 20 μm)

levels. This strategy was used in tomato, where the overexpression of *Escherichia coli* 1-deoxy-D-xylulose-5-phosphate synthase gene (*DXS*) resulted in a 1.6-fold increase in total fruit carotenoids (Enfissi et al. 2005). A second strategy involves the overexpression of carotenogenic genes in order to relieve bottlenecks in the pathway, thus increasing carotenoid levels and/or changing the composition. For example, the overexpression of bacterial phytoene synthase gene (*CRTB*) or corn phytoene synthase 1 gene (*PSY1*) (phytoene synthase, the first committed step in carotenoid biosynthesis) achieved a 50-fold increase in total carotenoid levels in Golden canola and a 156-fold increase in high-carotenoid corn (Shewmaker et al. 1999; Zhu et al. 2008). Carotenogenic gene expression can also be suppressed to block specific pathway branches, or to prevent the conversion of desirable compounds into downstream metabolites. For example, the downregulation of lycopene ε-cyclase gene (*LYCE*) in rapeseed blocked the carotenoid α-branch and diverted flux into the β-branch, increasing the seed carotenoid content by 45-fold (Yu et al. 2008). It is also possible to extend the pathway to produce novel compounds that are not usually synthesized in plants, e.g., the accumulation of 91 μg/g fresh weight (FW) of the ketocarotenoid astaxanthin in carrot taproots was achieved by expressing the *Haematococcus pluvialis* β-carotene ketolase gene (*BKT*) (Jayaraj et al. 2008).

Carotenoid levels can also be modulated by creating a metabolic sink, i.e., promoting the formation of carotenoid-sequestering structures (Fig. 1). This process is induced by the *orange* (*or*) gene (Li et al. 2001; Lu et al. 2006; Paolillo et al. 2004) originally discovered as a spontaneous dominant mutation in cauliflower (*Brassica oleracea* var.

botrytis) which resulted in orange curds (Crisp et al. 1975). In potato, expression of the dominant *or* allele of the cauliflower gene resulted in a tenfold increase in total carotenoids (Lopez et al. 2008). However, *or* did not influence metabolic flux through the cauliflower carotenoid pathway, nor did it affect the regulation of carotenogenic genes (Li et al. 2001). The plastid formation/translocation factor gene (*PFTF*) is upregulated in the *or* mutant, and the product of *or* is a plastid-associated protein with a DnaJ cysteine-rich domain, suggesting it may promote carotenoid accumulation by inducing the differentiation of proplastids into chromoplasts (Lu et al. 2006). In addition, the *or* mutation limits plastid replication so that the cell contains a single chromoplast, and orange crystalloid inclusions within this structure resemble the carotenoid inclusions found in carrot taproots (Paolillo et al. 2004).

The expression of cauliflower *or* in potato confirmed that the protein induces chromoplast differentiation and carotenoid sequestration, resulting in a sixfold increase in total carotenoids (Lopez et al. 2008). The only carotenoids present in wild-type tubers are violaxanthin and lutein, but the *or* transgenic tubers accumulated precursor carotenoids that are not detectable in wild-type tubers (such as phytoene, phytofluene, and ζ-carotene) as well as β-carotene, the latter representing 12–17% of the total carotenoids (Lopez et al. 2008). Carotenoids in the *or* transgenic lines remained stable for up to 6 months in cold storage leading to further accumulation, i.e., an 18-fold increase at 5 months and a 13-fold increase at 6 months (Li et al. 2012; Lopez et al. 2008). The co-expression of cauliflower *or* and *Brevundimonas* sp. SD212 *CRTZ* and *CRTW* increased the ketocarotenoid content of potato tubers (Campbell et al. 2015).

Orthologs of cauliflower wild-type *OR* have been identified in other plants and have been shown to influence carotenoid accumulation. For example, the sweet potato ortholog *IbOR* and the *Arabidopsis thaliana* ortholog *AtOR* can induce carotenoid accumulation in sweet potato callus and rice callus and plants (Kim et al. 2013; Bai et al. 2014, 2016). *IbOR* was shown to induce the expression of carotenoid biosynthesis genes as well as *PFTF* in transgenic sweet potato callus and the total carotenoid content increased by up to 12-fold (Kim et al. 2013). The total carotenoid content of rice callus expressing corn *PSY1* and *Pantoea ananatis CRTI* increased by 2.2-fold when *AtOR* was also expressed, and the endosperm contained orange crystalloid inclusions similar to those observed in transgenic potato plants expressing cauliflower *or* (Bai et al. 2014). Interestingly, similar structures were also observed in transgenic callus co-expressing *AtDXS, ZmPSY1*, and *PaCRTI*, suggesting that orange crystalloid inclusions in rice may be triggered by the abundance of carotenoids (Fraser et al. 2007; Maass et al. 2009; Bai et al. 2014). Similarly, plastids containing plastoglobuli were observed in rice endosperm overexpressing *AtOR* but in this case the expression of the endogenous carotenogenic genes *LYCE, LYCB*, and *BCH2* was also upregulated (Bai et al. 2016).

White corn (*Zea mays* cv. M37W) serves as a blank canvas for the analysis of carotenoid biosynthesis because the white endosperm contains only trace amounts of carotenoids due to the minimal expression of *ZmPSY1* and *ZmPSY2*. We generated M37W corn lines expressing *AtOR*, resulting in a substantial increase in the total carotenoid content as shown by the yellow endosperm color, but the increase was not accompanied by a qualitative change in the profile of endosperm carotenoids. The best-performing *AtOR* transgenic line was crossed with four different transgenic corn lines expressing distinct sets of carotenogenic genes and accumulating different carotenoid profiles, including ketocarotenoids. The resulting hybrids were used to determine the impact of *AtOR* on carotenoid metabolism and accumulation at the transcriptional and metabolic levels.

Results

Corn lines expressing *AtOR* accumulate more carotenoids than wild-type plants but the expression of endogenous carotenogenic genes is mostly unchanged

Immature embryos from the white corn inbred M37W were transformed with a construct carrying the *AtOR* gene under the control of the endosperm-specific wheat low molecular-weight glutenin promoter and another construct carrying the selectable marker *BAR* under the control of the constitutive corn *UBI-1* promoter. Two representative lines (OR1 and OR2) expressing *AtOR* at low and high levels, respectively, were selected for detailed analysis. Primary transformants were self-pollinated to homozygosity, and *AtOR* mRNA levels were measured by northern blot analysis in the endosperm of T_2 homozygous plants 30 days after pollination (DAP) (Supplementary Fig. 1).

Wild-type M37W endosperm accumulated only traces of zeaxanthin, lutein, violaxanthin, and antheraxanthin, with a total carotenoid content of ~1 µg/g dry weight (DW). In contrast, the endosperm of line OR1 accumulated higher levels of zeaxanthin (~6 µg/g DW) and lutein (~2 µg/g DW), as well as traces of β-cryptoxanthin, antheraxanthin, and violaxanthin. Line OR2 accumulated much higher levels of zeaxanthin (~10 µg/g DW) as well as significant amounts of lutein (~2 µg/g DW), antheraxanthin (~2 µg/g DW), and β-cryptoxanthin (~1 µg/g DW), and traces of violaxanthin. The total carotenoid contents of the endosperm in lines OR1 and OR2 were ~9 and ~17 µg/g DW, respectively (Fig. 2a). Line OR2 therefore achieved a 20-fold

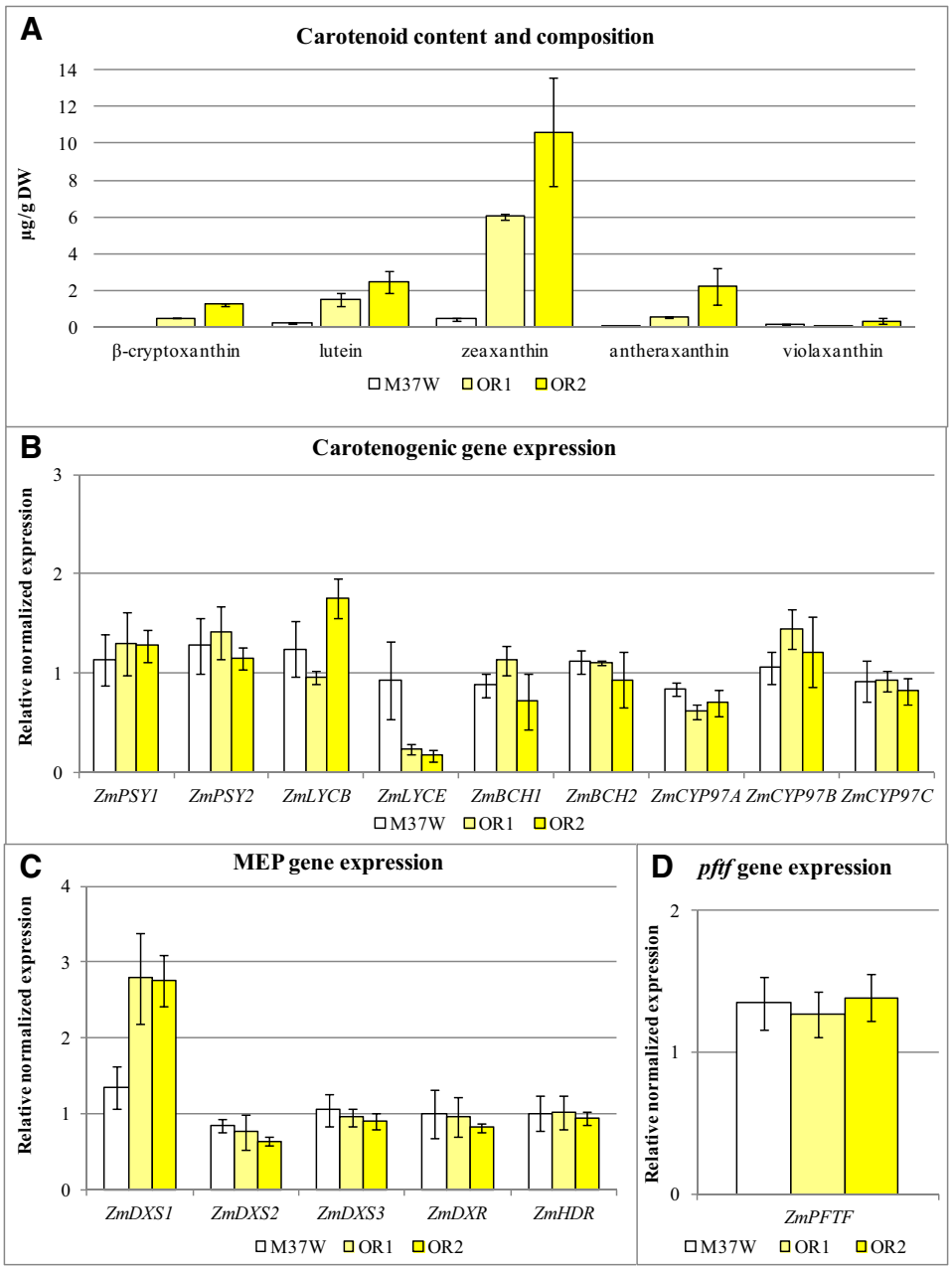

Fig. 2 **a** Carotenoid content and composition in wild-type M37W and transgenic lines OR1 and OR2 (T_2 plants) at 30 DAP (µg/g DW ± SE) (n = 3–5 seeds). Relative expression levels of endogenous carotenogenic genes (**b**) MEP pathway-related genes (**c**) and *PFTF* (**d**) in 30 DAP corn endosperm, normalized against *ACTIN* mRNA and presented as the mean of three biological replicates ± SE. *ZmPSY1* phytoene synthase 1, *ZmPSY2* phytoene synthase 2, *ZmLYCE* lycopene ε-cyclase, *ZmLYCB* lycopene β-cyclase, *ZmBCH1* carotenoid β-hydroxylase 1, *ZmBCH2* carotenoid β-hydroxylase 2, *ZmCYP97A/B* carotene β-hydroxylase, *ZmCYP97C* carotene ε-hydroxylase, *ZmDXS1/2/3* 1-deoxy-D-xylulose-5-phosphate synthase, *ZmDXR* 1-deoxy-D-xylulose-5-phosphate reductase, *ZmHDR* 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, *ZmPFTF* plastid fusion/translocation factor

increase in carotenoids compared to wild-type plants under greenhouse conditions.

The transcript levels of endogenous carotenogenic genes, MEP pathway genes, and *PFTF* were compared in lines OR1, OR2, and wild-type M37W by quantitative RT-PCR. The following carotenogenic genes were analyzed: *ZmPSY1* and *ZmPSY2, ZmLYCB* (lycopene β-cyclase), *ZmLYCE, ZmBCH1, ZmBCH2, ZmCYP97A*, and *ZmCYP97B* (carotene β-hydroxylase) and *ZmCYP97C* (carotenoid ε-hydroxylase). Most of the genes were expressed at similar levels in the transgenic and wild-type lines—the only exception was *ZmLYCE*, which was downregulated in both transgenic lines (Fig. 2b). The following MEP pathway genes were analyzed: *ZmDXS1, ZmDXS2*, and *ZmDXS3* (1-deoxy-D-xylulose-5-phosphate synthase 1, 2 and 3), *ZmDXR* (1-deoxy-D-xylulose-5-phosphate reductase), and *ZmHDR* (4-hydroxy-3-methylbut-2-enyl diphosphate reductase). Most of the genes were expressed at similar levels in the transgenic and wild-type lines—the only exception was *ZmDXS1*, which was upregulated by ~two-fold in the transgenic lines (Fig. 2c). The expression of *ZmPFTF* did not differ significantly among the three lines (Fig. 2d).

Introgression of *AtOR* increases the carotenoid content of hybrids with low carotenoid parents

The influence of *AtOR* on carotenoid accumulation was investigated in detail by crossing the best-performing *AtOR* line (OR2) with four transgenic lines characterized by different genetic backgrounds and different carotenoid profiles. Two of the lines were selected because they accumulate moderate (CARO1) or high (CARO2) levels of carotenoids and another two were selected because they accumulate low (KETO1) or high (KETO2) levels of ketocarotenoids. CARO1 (*ZmPSY1*) and KETO1 (*sBrCRTZ, sCrBKT*, and s*BrCRTW*) were produced via the same procedure used to generate the *AtOR* lines, whereas CARO2 (*ZmPSY1, PaCRTI*, and *GlLYCB*) and KETO2 (*ZmPSY1, sCrBKT, sBrCRTZ*, and *LYCE*-RNAi) were described previously (Zhu et al. 2008; Farré et al. 2016). Line OR2 was used as a pollen donor to generate hybrids ORxCARO1, ORxCARO2, ORxKETO1, and ORxKETO2 (Fig. 3).

The carotenoid content and composition of wild-type M37W, the five transgenic parental lines (OR2, CARO1, CARO2, KETO1, and KETO2), and the four hybrids (ORxCARO1, ORxCARO2, ORxKETO1, and ORxKETO2) were evaluated at 30 and 60 DAP to identify any changes in carotenoid content and composition at two different developmental stages, i.e., mid grain filling and mature seeds. Transgene expression in the parental lines and hybrids was measured by quantitative RT-PCR (Supplementary Fig. 2). As expected, the wild-type endosperm did not express any of the transgenes, and OR2 expressed *AtOR* alone. CARO1 expressed only *ZmPSY1*; CARO2 expressed *ZmPYS1, PaCRTI*, and *GlLYCB*; KETO1 expressed *sBrCRTZ*, s*CrBKT*, and s*BrCRTW*; and KETO2 expressed *ZmPSY1, LYCE*-RNAi, *sCrBKT*, and *sBrCRTZ*. The hybrid lines expressed *AtOR* as well as the appropriate transgene complement from the other parent (Supplementary Fig. 2).

Wild-type M37W at 30 DAP (Fig. 3A) accumulated low levels of zeaxanthin (~0.4 µg/g DW), lutein (~0.2 µg/g DW), and violaxanthin (0.1 µg/g DW) as well as traces of antheraxanthin. At 60 DAP, we detected zeaxanthin (~0.3 µg/g DW) and lutein (~0.2 µg/g DW) but no violaxanthin. When *AtOR* was expressed in this background, the endosperm turned yellow (Fig. 3B). The carotenoid profile in the endosperm of line OR2 remained the same as M37W but the total carotenoid content increased dramatically at 30 DAP from <1 µg/g DW in M37W to ~25 µg/g DW (a 32-fold increase). The levels of individual carotenoids increased to different degrees compared to the wild-type plants: zeaxanthin (~12 µg/g DW, 30-fold increase), antheraxanthin (~7 µg/g DW, 170-fold increase), lutein (~5 µg/g DW, 23-fold increase), and violaxanthin (~2 µg/g DW, 15-fold increase). At 60 DAP, the total carotenoid content was 20 µg/g DW. The downstream compounds violaxanthin and antheraxanthin could no longer be detected, but β-cryptoxanthin was detectable (~1 µg/g DW) and the principal carotenoids were zeaxanthin (~11 µg/g DW, 38-fold increase over wild type) and lutein (~8 µg/g DW, 39-fold increase over wild type) (Supplementary Table 1).

The endosperm of parental line CARO1 was orange (Fig. 3C). At 30 DAP, this accumulated ~66 µg/g DW in total carotenoids (94-fold increase over wild type) and at 60 DAP this increased to 91 µg/g DW (181-fold increase

Fig. 3 Phenotype of wild-type (**A**) and transgenic seeds with different carotenoid and ketocarotenoid profiles: OR2 (**B**), CARO1 (**C**), CARO2 (**E**), KETO1 (**G**), and KETO2 (**I**), and the resulting seeds from the cross with OR2: ORxCARO1 (**D**), ORxCARO2 (**F**), ORxKETO1 (**H**), and ORxKETO2 (**J**). A substantial change in seed color phenotype resulted when *AtOR* was expressed in the M37W background (*white* to *yellow*) and in low-ketocarotenoid (KETO1) background (*light pink* to *reddish-pink*) suggesting that *AtOR* plays an important role in carotenoid and ketocarotenoid accumulation when the amounts of carotenoids are low in the original (parent) line (KETO1). No changes in color phenotype were observed when the amounts of carotenoids are already high in the parents (CARO1, CARO2, and KETO2)

over wild type). At 30 DAP, the principal carotenoids were zeaxanthin (~23 µg/g DW; 58-fold increase over wild type), antheraxanthin (~14 µg/g DW; 350-fold increase over wild type), lutein (~9 µg/g DW; 47-fold increase over wild type), and phytoene (~8 µg/g DW), followed by violaxanthin (~4 µg/g DW; 39-fold increase over wild type), β-carotene (~3 µg/g DW), lycopene (~3 µg/g DW), and β-cryptoxanthin (~1 µg/g DW). By 60 DAP, the carotenoid composition had shifted upstream, with a larger increase in phytoene (~61 µg/g DW). The other principal carotenoids were zeaxanthin (~12 µg/g DW; 39-fold increase over wild type), lutein (~6 µg/g DW; 32-fold increase over wild type), and antheraxanthin (~6 µg/g DW), followed by β-carotene (~2 µg/g DW), violaxanthin (~2 µg/g DW), β-cryptoxanthin (~1 µg/g DW), and lycopene (~1 µg/g DW) (Supplementary Table 1). The endosperm of the ORxCARO1 hybrid was similar in color to the CARO1 parent (Fig. 3D). The carotenoid content and composition was also similar to CARO1, with a total carotenoid content at 30 DAP of ~68 µg/g DW. Adverse weather prevented the analysis of ORxCARO1 at 60 DAP (Supplementary Table 1).

The endosperm of parental line CARO2 was also orange (Fig. 3E) but it had a higher carotenoid content than CARO1, i.e., ~79 µg/g DW at 30 DAP (113-fold increase over wild type) and ~155 µg/g DW at 60 DAP (311-fold increase over wild type). At 30 DAP, the principal carotenoids were zeaxanthin (~23 µg/g DW; 58-fold increase over wild type), phytoene (~18 µg/g DW), antheraxanthin (~13 µg/g DW; 330-fold increase over wild type), β-carotene (~11 µg/g DW), and lutein (~9 µg/g DW; 47-fold increase over wild type), followed by violaxanthin (~2 µg/g DW; 24-fold increase over wild type) and β-cryptoxanthin (~2 µg/g DW). As discussed above for CARO1, the phytoene content had increased (~117 µg/g DW) at the expense of downstream carotenoids by 60 DAP. The principal carotenoids were zeaxanthin (~18 µg/g DW; 60-fold increase over wild type), lutein (~6 µg/g DW; 44-fold increase over wild type), and β-carotene (~8 µg/g DW), followed by antheraxanthin (~2 µg/g DW) and β-cryptoxanthin (~2 µg/g DW) (Supplementary Table 1). The endosperm of the ORxCARO2 hybrid was orange, similar to the CARO2 parent (Fig. 3F). The carotenoid content and composition in the hybrid was similar to CARO2, with a total carotenoid content of ~79 µg/g DW at 30 DAP and ~135 µg/g DW at 60 DAP (Supplementary Table 1).

The endosperm of the parental line KETO1 was pale pink (Fig. 3G) reflecting the accumulation of relatively low levels of the ketocarotenoid astaxanthin (~0.4 µg/g DW at 30 DAP and ~0.6 µg/g DW at 60 DAP) with undetectable levels of other carotenoids. The minimal carotenoid pool in the wild-type M37W endosperm was therefore converted almost entirely into ketocarotenoids by expressing the heterologous carotenoid β-hydroxylase (*sBrCRTZ*) and β-carotene ketolases (*sCrBKT* and *sBrCRTW*) (Supplementary Table 2). The endosperm of the ORxKETO1 hybrid was deeper pink (Fig. 3H) reflecting the accumulation of more ketocarotenoids than the KETO1 parent. However, the profile was the same, i.e., the total carotenoid content of ~9 µg/g DW at 30 DAP (22-fold increase over KETO1) and ~8 µg/g DW at 60 DAP (14-fold increase over KETO1) was almost entirely due to the accumulation of astaxanthin (Supplementary Tables 1 and 2).

The endosperm of the parental line KETO2 was red (Fig. 3I) reflecting the higher levels of ketocarotenoids compared to KETO1, i.e., ~32 µg/g DW at 30 DAP (80-fold increase over KETO1) and ~26 µg/g DW at 60 DAP (17-fold increase over KETO1). KETO2 also accumulated other (non-keto) carotenoids (~33 µg/g DW at 30 DAP and ~79 µg/g DW at 60 DAP). At 30 DAP, the principal carotenoid was astaxanthin (~22 µg/g DW), accompanied by lower amounts of the other ketocarotenoids adonirubin (~3 µg/g DW), 3-OH-echinenone (~3 µg/g DW), canthaxanthin (~2 µg/g DW), and adonixanthin (~2 µg/g DW). The KETO2 endosperm at 30 DAP also accumulated lutein (~1 µg/g DW; sixfold increase over wild type), zeaxanthin (~8 µg/g DW; 19-fold increase over wild type), β-carotene (~7 µg/g DW), antheraxanthin (~5 µg/g DW; 113-fold increase over wild type), β-cryptoxanthin (~3 µg/g DW), and violaxanthin (~1 µg/g DW; eightfold over wild type), as well as the upstream, precursors lycopene (~5 µg/g DW) and phytoene (~4 µg/g DW). At 60 DAP, the endosperm of line KETO2 followed the trend observed in CARO1 and CARO2 by accumulating higher amounts of the upstream precursor phytoene (~54 µg/g DW) at the expense of downstream carotenoids. Astaxanthin was still the predominant ketocarotenoid at 60 DAP (~16 µg/g DW), followed by adonirubin (~4 µg/g DW), canthaxanthin (~3 µg/g DW), 3-OH-echinenone (~2 µg/g DW), and adonixanthin (~1 µg/g DW) (Supplementary Table 2). The KETO2 endosperm at 60 DAP also accumulated β-carotene (~12 µg/g DW), zeaxanthin (~6 µg/g DW; 21-fold increase over wild type), and ~1 µg/g DW lutein, the latter much less abundant than in CARO1 and CARO2 (Supplementary Table 1). The hybrid ORxKETO2 had a similar red endosperm color to the KETO2 parent (Fig. 3j) and the carotenoid profile was also similar. ORxKETO2 accumulated ~30 µg/g DW total carotenoids (28 µg/g DW ketocarotenoids) at 30 DAP and ~74 µg/g DW total carotenoids (16 µg/g DW ketocarotenoids) at 60 DAP (Supplementary Tables 1 and 2).

Endogenous carotenoid pathway, MEP pathway, and *PFTF* gene expression is similar in the hybrids and their parents

The expression of endogenous carotenogenic genes, MEP pathway genes, and *PFTF* was compared in lines CARO2,

KETO2, the corresponding hybrids, and wild-type M37W plants by quantitative RT-PCR (Table 1).

In line CARO2, most of the endogenous genes were expressed at the same level as wild-type line. Four genes were expressed at higher levels in the transgenic plants: *ZmCYP97A* (twofold), *ZmBCH1* (threefold), and *ZmLYCB* (sixfold), representing the carotenoid pathway, and *ZmDXS2* (11-fold) representing the MEP pathway. Furthermore, the *PSY1* transcript was ~10,000-fold more abundant in line CARO2 because this was the transgene product. In hybrid ORxCARO2, *ZmCYP97A* expression was similar to wild-type levels, thus lower than CARO2, and *ZmBCH1* expression was similar to CARO2, thus threefold higher than wild type. Two further genes were expressed at higher levels than the wild-type plants but at lower levels than CARO2 (*ZmLYCB*, ~twofold higher than wild type, and *ZmDXS2*, sevenfold higher than wild type). Two genes were expressed at higher levels than in either of the parental lines (*ZmBCH2*, ~threefold higher than wild type, and *ZmDXR*, ~twofold higher than wild type). As in line CARO2, the *ZmPSY1* transcript was much more abundant than in wild-type plants (~5600-fold increase) due to transgene expression.

In KETO2, most of the endogenous genes were expressed at the same level as wild type but six genes were expressed at higher levels in the transgenic plants: *ZmLYCB, ZmBCH1, ZmCYP97A, ZmCYP97B, ZmCYP97C*, and *ZmDXS2* (all ~fourfold). As above, the *ZmPSY1* transcript was more abundant (~1000-fold) in the transgenic line due to transgene expression. In hybrid ORxKETO2, *ZmLYCE, ZmBCH2, ZmDXS1, ZmDXS3, ZmDXR*, and *ZmPFTF* were expressed at wild-type levels. The *ZmCYP97A* and *ZmCYP97B* transcripts were wild-type levels and were therefore less abundant than in the KETO2 parent, whereas the *ZmPSY1, ZmPYS2, ZmLYCB, ZmBCH2, ZmHDR*, and *ZmDXS2* transcripts were present at similar levels to the KETO2 parent and were therefore more abundant than in wild-type plants. The *ZmCYP97C* transcript was present at similar levels in KETO2, ORxKETO2, and wild-type plants.

In KETO1, most of the endogenous genes were expressed at the same level as wild type but *ZmLYCE* and *ZmBCH1* were downregulated by up to threefold in the transgenic line and similar profiles were observed for most genes in the hybrid ORxKETO1. There were two exceptions: *ZmDXS1* mRNA accumulated at significantly higher levels in the hybrid line compared to wild-type plants, and *ZmDXR* levels remained similar to wild-type levels but were significantly higher compared to KETO1 plants (Table 1).

Table 1 Relative expression levels of endogenous carotenogenic genes, MEP pathway-related genes, and PFTF in 30 DAP corn endosperm, normalized against *ACTIN* mRNA and presented as the mean of three biological replicates

Genes	Wt	CARO2	ORxCARO2	KETO2	ORxKETO2	KETO1	ORxKETO1
ZmPSY1	1.00d	9940a	5585b	1027c	927c	1.08d	1.10d
ZmPSY2	1.00b	0.79b	1.20b	1.48ab	2.34a	1.03b	1.05b
ZmLYCE	1.00ab	1.23ab	1.91a	0.83b	1.06ab	0.35c	0.45c
ZmLYCB	1.00c	5.94a	2.35b	3.21ab	2.34b	0.85c	0.77c
ZmBCH1	1.00c	2.95b	2.11b	4.34a	4.51a	0.52d	0.64d
ZmBCH2	1.00b	1.53a	2.55a	1.86ab	1.75ab	1.43b	1.38b
ZmCYP97A	1.00b	1.60a	1.10b	1.61a	1.09b	1.24b	1.16b
ZmCYP97B	1.00b	1.34b	2.16ab	3.25a	2.17ab	1.15b	1.28b
ZmCYP97C	1.00b	1.26b	1.37ab	2.20a	1.51ab	1.09b	1.16b
ZmDXS1	1.00b	1.01b	0.68b	0.81b	0.67b	0.83b	1.40a
ZmDXS2	1.00c	11.13a	6.74b	3.79b	4.35b	0.72c	0.86c
ZmDXS3	1.00a	0.90a	1.21a	0.94a	1.80a	0.69a	0.75a
ZmDXR	1.00b	1.06b	1.64a	1.05b	0.83b	0.42c	1.08b
ZmHDR	1.00b	1.80ab	2.27ab	2.92ab	3.22a	1.13b	1.06b
ZmPFTF	1.00a	0.90a	1.08a	1.02a	1.28a	1.35b	1.10b

Transcript levels in wild-type M37W plants (WT) were used as a reference and assigned the value 1.00

Different letters correspond to statistically significant differences between groups ($p < 001$). Means not sharing the same letter are significantly different (Tukey HSD, $p < 0.05$)

ZmPSY1 phytoene synthase 1, *ZmPSY2* phytoene synthase 2, *ZmLYCE* lycopene ε-cyclase, *ZmLYCB* lycopene β-cyclase, *ZmBCH1* carotenoid β-hydroxylase 1, *ZmBCH2* carotenoid β-hydroxylase 2, *ZmCYP97A/B* P450-carotenoid β-hydroxylase, *ZmCYP97C* P450-carotenoid ε-hydroxylase, *ZmDXS1/2/3* 1-deoxy-D-xylulose-5-phosphate synthase, *ZmDXR* 1-deoxy-D-xylulose-5-phosphate reductase, *ZmHDR* methylbut-2-enyl diphosphate reductase, *ZmPFTF* plastid fusion/translocation factor

Higher carotenoid levels in diverse genetic backgrounds promote the creation of a metabolic sink

Transmission electron microscopy (TEM) was used to analyze of the first layer of endosperm cells under the epithelium of wild type, OR2, CARO1, CARO2, KETO1, KETO2, and ORxKETO1 plants at 30 DAP. This revealed electron-dense plastoglobuli inside the plastids. These were scarce in the wild-type and KETO1 lines, which accumulate only low levels of total carotenoids, but much more abundant in the CARO1, CARO2, and KETO2 lines, which have a high-carotenoid content. There were also more plastoglobuli in the OR2 and ORxKETO1 lines compared to the wild-type and KETO1 lines, respectively (Fig. 4).

Discussion

The overexpression of *AtOR* enhances total carotenoid levels in hybrids of parents with a restricted carotenoid pool

A splicing mutation in the cauliflower *orange* (*or*) gene yields a dominant allele (*or*) that increases carotenoid (particularly β-carotene) accumulation by promoting chromoplast differentiation and creating a metabolic sink (Li et al. 2001). The function is transferable to other plants, e.g., transgenic potato (cv. Desiree) tubers expressing cauliflower *or* also accumulated β-carotene and other carotenoids, with progressive accumulation during cold storage (Lopez et al. 2008; Li et al. 2012). Interestingly, when the same approach was tested in a different potato variety (cv. Phureja), there was no increase in the carotenoid content during cold storage and the principal carotenoids that accumulated were zeaxanthin, antheraxanthin, violaxanthin, and lutein (Campbell et al. 2015). The distinct behavior of different potato cultivars suggests that endogenous carotenoid profiles may influence the manner in which *or* gene expression influences carotenoid accumulation in other species.

In rice, the overexpression of *AtOR* alone had no impact on carotenoid accumulation, but carotenoids accumulated to higher levels in callus (Bai et al. 2014) and seeds (Bai et al. 2016) when *AtOR* was co-expressed with *ZmPSY1* and *PaCRTI*. This suggested that chromoplast differentiation can be triggered by carotenoid accumulation above a certain threshold and that the presence of the OR protein may augment or potentiate this process but is not sufficient on its own to induce the formation of a metabolic sink (Bai et al. 2014). The sweet potato ortholog *IbOR* not only increased the β-carotene content of transgenic sweet potato callus, but also increased the levels of α-carotene, lutein, β-crytoxanthin, and zeaxanthin, suggesting that *IbOR* can influence carotenogenic

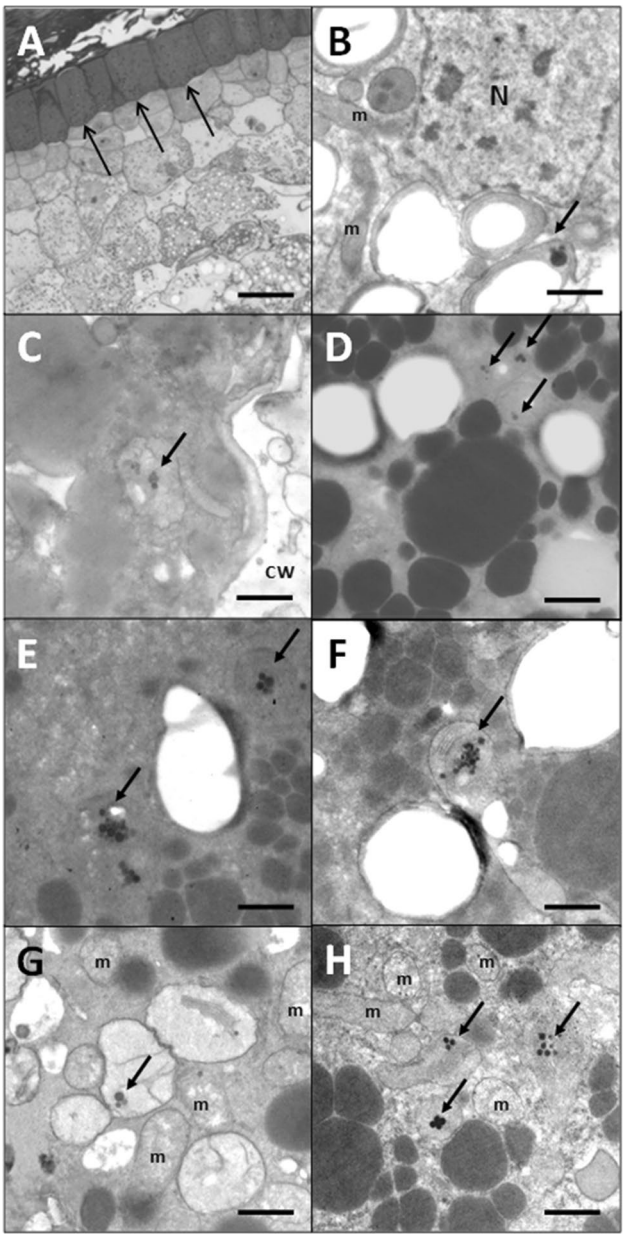

Fig. 4 Micrographs of 30 DAP endosperm from wild-type (WT) and transgenic corn lines OR2, CARO1, CARO2, KETO1, KETO2, and ORxKETO1. **A** Light micrograph of WT endosperm; *arrows* indicate aleurone cell layer (**B**–**H**). Transmission electron micrographs of WT (**B**), OR2 (**C**), CARO1 (**D**), CARO2 (**E**), KETO2 (**F**), KETO1 (**G**), and ORxKETO1 (**H**). Arrows indicate plastoglobuli inside plastids m mitochondria, cw cell wall, N nucleus. *Scale bar* 30 µm (**A**) and 0.7 µm (**B**–**H**)

gene expression. The transgenic *IbOR* callus also possessed higher antioxidant activity and was more tolerant to salt stress, probably reflecting the increase in carotenoid levels (Kim et al. 2013). The overexpression of *IbOR* in sweet potato plants increased the total carotenoid content by ~threefold although the composition was not affected, i.e., the wild-type distribution of

carotenoids was maintained but the abundance of each carotenoid increased (Goo et al. 2015). The same phenomenon was observed in our OR1 and OR2 lines, but in potato the expression of cauliflower *or* changed both the content and the composition of carotenoids (Lopez et al. 2008), indicating that the function of *or* may be context-specific, i.e., in some backgrounds it may influence gene expression and carotenoid composition, but in others it may only induce a sink for existing carotenoids thereby increasing the quantity without affecting composition. In corn hybrids, the impact of *AtOR* introgression depended on the nature of the carotenoid pool in the carotenogenic parental line. If the parental carotenoid pool was restricted, as was the case for line KETO1, the introgression of *AtOR* increased the total carotenoid content by up to 22-fold at 30 DAP in the hybrid, again without affecting the carotenoid profile (Supplementary Table 1). This suggests that *AtOR* enhances carotenoid accumulation in corn but does not alter the underlying metabolic fluxes by modulating the expression of carotenogenic genes in a substantial manner. The carotenoid composition of line KETO1 did not change substantially between 30 and 60 DAP and the ORxKETO1 hybrid behaved in a similar manner, indicating that carotenoid levels remain relatively constant throughout seed development in this line (Fig. 5A). In contrast, when the carotenoid pool in the parent was abundant (i.e., above 70 µg/g DW in the case of CARO1, CARO2, and KETO2), *AtOR* had no influence on either the carotenoid content or the carotenoid profile at 30 or 60 DAP in the corresponding hybrids. The carotenoid composition of lines CARO1, CARO2, and KETO2 changed between 30 and 60 DAP mainly because of the increase in phytoene levels at the expense of downstream carotenoids such as lutein, zeaxanthin, and antheraxanthin. The behavior of the hybrids was similar to the carotenogenic parents (Fig. 5B). ORs from Arabidopsis (*Arabidopsis thaliana*), sweet potato (*Ipomoea batatas*), and melon (*Cucumis melo*) have been reported to stabilize PSY protein (Zhou et al. 2015; Park et al. 2016; Chayut et al. 2017). In white M37W maize, limited amounts of carotenoids accumulate in the endosperm due to low *ZmPSY1* and *ZmPSY2* mRNA accumulation. The interaction of *AtOR* with *ZmPSY1* and/or *ZmPSY2* might increase endogenous *ZmPSY1* and/or *ZmPSY2* protein levels via stabilization of PSY protein, resulting in increased carotenoid content in the endosperm of ORxKETO1 compared to KETO1. However, since *ZmPSY1* was overexpressed in the endosperm of ORxCARO1, ORxCARO2, and ORxKETO2 hybrids at levels similar in CARO1, CARO2, and KETO2 parental lines, respectively, any post-transcriptional regulation of *ZmPSY1* and/or *ZmPSY2* protein levels does not appear to cause substantial differences in carotenoid accumulation in the endosperm of CARO1 and ORxCARO1, CARO2 and ORxCARO2, or KETO2 and ORxKETO2.

AtOR has little impact on endogenous carotenoid and MEP pathway genes and *PFTF* despite the higher total carotenoid levels

The spontaneous cauliflower mutation *or* does not induce changes in endogenous carotenogenic gene expression (Li et al. 2001, 2006; Lu et al. 2006). The cauliflower OR protein has been shown to stabilize the enzyme phytoene synthase, enhancing its activity and allowing the continuous biosynthesis of carotenoids during storage (Zhou et al. 2015). The cauliflower OR protein was suggested to function in association with the molecular chaperone system to promote protein folding (Li et al. 2012). More recently, the AtOR protein has been shown to function as a major post-transcriptional regulator of phytoene synthase activity and can modulate carotenoid biosynthesis in this manner (Zhou et al. 2015). In contrast, the overexpression of *IbOR* in sweet potato callus increased carotenoid accumulation by inducing the endogenous carotenoid biosynthesis genes *IbPSY1, IbCRTISO, IbLYCB*, and *IbBCH* (Kim et al. 2013). Similarly, the overexpression of *IbOR* in an anthocyanin-rich purple-fleshed cultivar induced the carotenogenic genes *IbPDS, IbZDS, IbLYCB, IbBCH*, and *IbZEP*. Interestingly, *IbLYCE* expression was suppressed, whereas the genes encoding carotenoid cleavage dioxygenases (*IbCCD1* and *IbCCD4*) and 9-cis-epoxycarotenoid dioxygenases (*NCED*) were induced (Goo et al. 2015). The overexpression of *AtOR* in rice endosperm induced the endogenous carotenogenic genes *OsLYCE, OsLYCB*, and *OsBCH2* (Bai et al. 2016). These results suggested that *AtOR* may increase carotenoid levels in some backgrounds but not others by boosting endogenous carotenoid biosynthesis at the transcriptional level.

In our corn plants, the detailed analysis of carotenogenic gene expression revealed no dramatic changes in transcript levels between wild-type and *AtOR* transgenic lines, except the ~twofold downregulation of *ZmLYCE*, which correlates with the high zeaxanthin levels in the transgenic lines reflecting the redirection of flux from the α-branch to the β-branch of the pathway (Fig. 2). The downregulation of *ZmLYCE* was also observed in KETO1 (threefold decrease compared to wild type), suggesting that extending the carotenoid pathway to ketocarotenoids directs flux to the β-branch of the pathway. *ZmLYCE* was also downregulated when *AtOR* was introgressed into a more complex corn line expressing a carotenoid β-hydroxylase (*sBrCRTZ*) and two β-carotene ketolases (*sCrBKT* and *sBrCRTW*). *ZmLYCE* expression in the ORxKETO1 hybrid was similar to the OR2 and KETO1 parents (Table 1) but remained similar to wild-type levels in the CARO1, CARO2, and KETO2

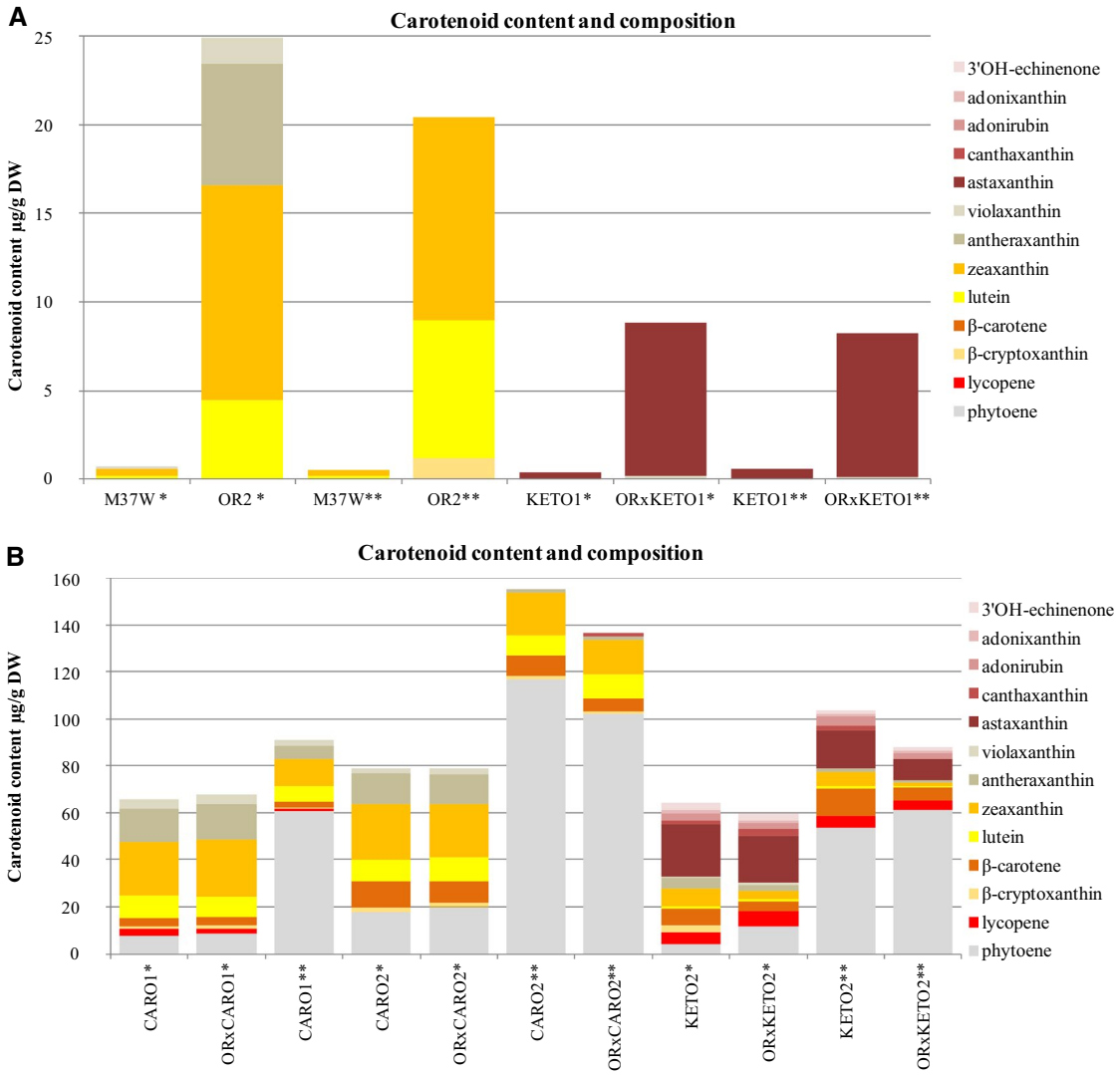

Fig. 5 **A** Total carotenoid content and composition in wild-type M37W, transgenic lines OR2 and KETO1, and hybrid ORxKETO1 in the T_1 generation at 30 DAP (*) and 60 DAP (**) (n = 3–5 seeds). **B** Carotenoid content and composition in CARO1, CARO2, and KETO2, and hybrids ORxCARO1, ORxCARO2, and ORxKETO2 in the T_1 generation at 30 DAP (*) and 60 DAP (**) (n = 3–5 seeds)

lines and their corresponding hybrids (ORxCARO1, ORxCARO2, and ORxKETO2), suggesting that *AtOR* does not affect endogenous carotenogenic gene expression in these lines. Furthermore, *ZmLYCB* was induced in the transgenic lines overexpressing carotenogenic genes (CARO1, CARO2, and KETO2) and their corresponding hybrids (ORxCARO1, ORxCARO2, and ORxKETO2) suggesting that the overexpression of carotenogenic genes in CARO1 (*ZmPSY1*), CARO2 (*ZmPSY1, PaCRTI*, and *GlLYCB*) and KETO2 (*ZmPSY1, sCRBKT*, and *sBRCRTZ*) directs flux through the β-branch of the pathway in the M37W background.

The influence of *AtOR* on early steps in the carotenoid pathway was investigated by analyzing the expression of endogenous MEP pathway genes (*ZmDXS1, ZmDXS2, ZmDXS3, ZmDXR*, and *ZmHDR*). No changes were detected in line OR2 except a ~twofold upregulation of *ZmDXS1*, suggesting that flux towards the carotenoid pathway increases slightly due to *AtOR*, thus promoting the biosynthesis of all carotenoids (Fig. 2). Similar results were observed when *AtOR* was introgressed into the hybrid ORxKETO1, but *ZmDXS1* transcript levels remained at wild-type levels in transgenic lines overexpressing carotenogenic genes (CARO1, CARO2 and KETO2) and their

corresponding hybrids (Table 1). However, *ZmDXS2* transcript levels increased in the same three lines and their hybrids, suggesting that the overexpression of carotenogenic genes causes positive feedback regulation and increases metabolic flux through the carotenoid pathway in the M37W background. In *A. thaliana* callus, *AtOR* marginally increased the expression of *AtDXS* (~1.2-fold) and *AtDXR* (~1.3-fold) compared to wild-type callus (Yuan et al. 2015).

The plastid fusion/translocation factor (PFTF) is required for chromoplast differentiation in red pepper (Hugueney et al. 1995). The overexpression of *IbOR* increased *PFTF* transcript levels in sweet potato callus and tubers, suggesting that *IbOR* can trigger chromoplast formation via PFTF (Kim et al. 2013; Goo et al. 2015). However, *PFTF* transcript levels were not affected in our corn hybrids expressing *AtOR* suggesting that *AtOR* does not trigger chromoplast formation by itself in corn (Fig. 5).

High levels of nascent carotenoids rather than *AtOR* gene expression trigger the formation of carotenoid-rich plastoglobuli in corn endosperm

Cauliflower *or* is the only known gene that acts as a bona fide molecular switch to trigger the differentiation of non-colored plastids into chromoplasts (Li et al. 2006, 2012; Lu et al. 2006). Experimental evidence to support the role of cauliflower *or* was provided by the formation of chromoplast-containing carotenoid-sequestering structures in potato tubers expressing the dominant allele *or*, whereas chromoplasts were not formed in potato cultivars accumulating high levels of carotenoids in the absence of *or* expression (Lopez et al. 2008). The replacement of a single amino acid in the wild-type *AtOR* sequence greatly enhanced its ability to promote carotenoid accumulation by triggering the biogenesis of membranous chromoplasts in *A. thaliana* callus without altering carotenogenic gene expression (Yuan et al. 2015). In contrast, morphological changes in plastids have been observed in tomato fruits and canola endosperm expressing phytoene synthase (Shewmaker et al. 1999; Fraser et al. 2007; Nogueira et al. 2013). These observations suggest that plastids are modified to accommodate higher levels of carotenoids. The overexpression of phytoene synthase can also induce crystalloid carotenoid-sequestering structures in *A. thaliana* callus, suggesting that the chromoplast differentiation program may respond to the accumulation of carotenoids above a certain threshold, unless differentiation is triggered by *AtOR* before this threshold is reached (Maass et al. 2009). In transgenic rice, *AtOR* expression might influence carotenoid levels directly as well as indirectly. Chromoplast differentiation is primarily triggered by carotenoid accumulation above a certain threshold and the presence of *AtOR* protein may augment and potentiate this process. However, the expression of *AtOR* alone in rice is not sufficient to trigger chromoplast differentiation (Bai et al. 2014). Storage organs in many species synthesize and deposit carotenoids primarily in the membranes of amyloplasts (Li et al. 2012), which sequester β-carotene in a crystalloid metabolic sink (Cao et al. 2012). These structures have two roles: first, they sequester excess carotenoids and keep them away from plastid membranes, thus stimulating continuous carotenoid biosynthesis, and second, they provide a stable metabolic sink that protects carotenoids from degradation (Li et al. 2012). Amyloplasts in corn contain carotenoids (Wurtzel 2004). In our experiments, we observed amyloplasts and other plastids with electron-dense plastoglobuli that might contain residual levels of carotenoids in a low-carotenoid genetic background (M37W and KETO1). However, more plastoglobuli were observed in high-carotenoid genetic backgrounds (CARO1, CARO2, and KETO2). Furthermore, there were fewer plastids containing plastoglobuli, as well as fewer plastoglobuli inside the plastids, in the OR2 and KETO1 lines with limited carotenoid pools, suggesting that plastoglobuli in corn are formed due to higher carotenoid levels. *AtOR* expression in *A. thaliana* callus does not change plastid morphology per se but a single amino acid substitution in *AtOR* yielded plastids with larger and more electron-dense plastoglobuli. The mutant AtOR triggered biogenesis of membranous chromoplasts in the Arabidopsis calli, which shared structures similar to chromoplasts found in the curd of the orange cauliflower (Brassica oleracea) mutant (Yuan et al. 2015).

In summary, the overexpression of *AtOR* in the white-endosperm inbred corn line M37W increased the carotenoid content of the endosperm without affecting the expression of endogenous carotenogenic or MEP pathway genes, except for the induction of *ZmDXS1*. Crossing the best-performing *AtOR* line with four different transgenic parental lines accumulating different levels of carotenoids and/or ketocarotenoids yielded hybrids with the same qualitative carotenoid profiles as their parents. The total carotenoid levels in the hybrid increased when the carotenoid pool in the parent was restricted but there was no significant change when carotenoids were already abundant. The overexpression of *ZmPSY1* alone or in combination with other carotenogenic genes induced the formation of plastoglobuli that provide a metabolic sink for carotenoids inside the plastids, and similar structures were observed in the original *AtOR* line. However, the abundance of the plastids and the plastoglobuli within them appeared to be dependent on the carotenoid content. It therefore appears that in corn, as previously shown in rice, unlike the cauliflower *orange* (*or*) gene mutant version, the *AtOR* gene is not sufficient on its own to induce the differentiation of proplastids into chromoplasts *en masse*.

Experimental procedures

Gene cloning and vector construction

Vectors carrying the *A. thaliana ORANGE* (*AtOR*) and *Chlamydomonas reinhardtii bkt* (*sCrBKT*) genes were previously described by Bai et al. (2014). Vectors carrying the *Brevundimonas* sp. SD212 (MBIC 03018) β-carotene hydroxylase gene (*sBrCRTZ*) and the RNAi construct to block lycopene ε-cyclase expression were previously described by Farré et al. (2016). Vectors carrying the *ZmPSY1, GlLYCB*, and *PaCRTI* genes were previously described by Zhu et al. (2008). The vector carrying the *Brevundimonas* sp. SD212 β-carotene ketolase (*sBrCRTW*) was previously described by Breitenbach et al. (2014). The vectors are shown in Supplementary Fig. 3.

Corn transformation and plant growth

Transgenic corn plants expressing *AtOR* (lines OR1 and OR2); *ZmPSY1* (line CARO1), *ZmPSY1, PaCRTI*, and *GlLYCB* (line CARO2); *sBrCRTZ, sBrCRTW*, and *sCrBKT* (line KETO1); and *ZmPSY1, LYCE-RNAi, sCrBKT*, and *sBrCRTZ* (line KETO2) were generated as previously described (Zhu et al. 2008; Farré et al. 2016). The best-performing line expressing each transgene combination was selected by RNA blot analysis as previously described (Zhu et al. 2008; Farré et al. 2016). Each line was self-pollinated to homozygosity and used as a parental line for hybridization. The CARO1, CARO2, KETO1, and KETO2 lines were outcrossed with an OR pollen donor to obtain hybrids ORxCARO1, ORxCARO2, ORxKETO1, and ORxKETO2. For further analysis, endosperm samples were taken from immature seeds at 30 and 60 DAP, frozen in liquid nitrogen, and stored at −80 °C.

Synthesis of cDNA and real-time quantitative RT-PCR

The synthesis of cDNA and real-time quantitative RT-PCR were carried out as reported by Naqvi et al. (2011) using the primers listed in Supplementary Table 3.

Carotenoid extraction and analysis

Corn endosperm was excised by removing the seed coat and embryo. Samples were freeze-dried before extraction, and 3–5 seeds per sample were ground to a fine powder. Samples of 50–100 mg were extracted in 15 ml methanol:ethyl acetate (6:4 v/v) at 58 °C for 20 min. The mixture was filtered, transferred to a funnel, and 15 ml hexane:diethyl ether (9:1 v/v) was added followed by gentle agitation for 1 min. The organic phase was washed twice with saturated NaCl, and the aqueous phase was removed. The samples were dried under N_2 and stored at −80 °C.

The extracts were dissolved in 210–600 µl an injection solvent comprising three volumes of acetonitrile/methanol (7:3 v/v) and two volumes of acetone. The extracted carotenoids were separated by ultra-high performance liquid chromatography (UHPLC) at SCT-DATCEM (University of Lleida, Spain) using an ACQUITY Ultra Performance LC system linked to a PDA 2996 detector (Waters, Milford, USA). Mass detection was carried out using an ACQUITY TQD tandem-quadrupole MS equipped with a Z-spray electrospray interface (Waters). MassLynx software v4.1 (Waters) was used to control the instruments and also for data acquisition and processing. UHPLC separations were performed on a reversed-phase ACQUITY UPLC C18 BEH 130 Å, 1.7 µm, 2.1 × 150 mm column (Waters). The mobile phase consisted of solvent A (acetonitrile:methanol, 7:3 v/v) and solvent B (100% water). Carotenoids were quantified using a PDA detector and the external standard method (Rivera et al. 2013). MS analysis was conducted by atmospheric pressure chemical ionization (APCI) as described by Rivera et al. (2011). The following authentic standards were used for quantification: β-carotene, lutein, β-cryptoxanthin, and astaxanthin (Sigma, St Louis, MO, USA); zeaxanthin (Fluka, Buchs SG, Switzerland); phytoene and antheraxanthin (Carotenature, Lupsingen, Switzerland).

Transmission electron microscopy

Corn endosperm at 30 DAP (0.5 × 2.0 mm pieces) was processed as described by Bai et al. (2014).

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Author's contribution CZ, GS, TC, and PC designed research; JB, UZ, and GF performed research, analyzed data, and wrote the article; VM did TEM and analyzed these data.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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