Engineered Maize Hybrids with Diverse Carotenoid Profiles and Potential Applications in Animal Feeding

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Abstract

Multi-gene transformation methods need to be able to introduce multiple transgenes into plants in order to reconstitute a transgenic locus where the introduced genes express in a coordinated manner and do not segregate in subsequent generations. This simultaneous multiple gene transfer enables the study and modulation of the entire metabolic pathways and the elucidation of complex genetic control circuits and regulatory hierarchies. We used combinatorial nuclear transformation to produce multiplex-transgenic maize plants. In proof of principle experiments, we co-expressed five carotenogenic genes in maize endosperm. The resulting combinatorial transgenic maize plant population, equivalent to a "mutant series," allowed us to identify and complement rate-limiting steps in the extended endosperm carotenoid pathway and to recover corn plants with extraordinary levels of β-carotene and other nutritionally important carotenoids. We then introgressed the induced (transgenic) carotenoid pathway in a transgenic line accumulating high levels of nutritionally important carotenoids into a wild-type yellow-endosperm variety with a high β:ε ratio. Novel hybrids accumulated zeaxanthin at unprecedented amounts. We introgressed the same pathway into a different yellow corn line with a low β:ε ratio. The resulting hybrids, in this case, had a very different carotenoid profile. The role of genetic background in determining carotenoid profiles in corn was elucidated, and further rate-limiting steps in the pathway were identified and resolved in hybrids. Astaxanthin accumulation was engineered by overexpression of a β-carotene ketolase in maize endosperm. In early experiments, limited astaxanthin accumulation in transgenic maize plants was attributed to a

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bottleneck in the conversion of adonixanthin (4-ketozeaxanthin) to astaxanthin. More recent experiments showed that a synthetic β-carotene ketolase with a superior β-carotene/zeaxanthin ketolase activity is critical for the high-yield production of astaxanthin in maize endosperm. Engineered lines were used in animal feeding experiments which demonstrated not only the safety of the engineered lines but also their efficacy in a range of different animal production applications.

Keywords

Maize (*Zea mays*) · Carotenoids · β-Carotene · Multi-gene transformation · Transgene

8.1 Introduction

Carotenoids are high-value industrially important products, used as food colorants, and are essential in the human diet (Naqvi et al. 2011a). Different carotenoids have different functions (Bai et al. 2011; Farre et al. 2010, 2011; Berman et al. 2015). Four carotenoids (β-carotene, α-carotene, γ-carotene, and β-cryptoxanthin) have vitamin A activity in humans, which means that they can be converted into the visual pigment retinal and are classified as essential nutrients. Vitamin A plays an important role in the human body for normal growth and tissue repair. The visual and immune systems are particularly dependent on this vitamin for normal function. Lycopene is the red pigment in many fruits and vegetables, such as tomato and watermelon, and it does not have pro-vitamin A activity; however, it is an excellent dietary antioxidant and plays a role in reducing the risk of a number of cancers and coronary heart disease. Lutein and zeaxanthin constitute the major carotenoids of the yellow spot in the human retina and protect against age-related macular degeneration, which is the main cause of blindness in elderly people in the industrialized world. Astaxanthin helps prevent certain types of cancer, inhibits the oxidation of low-density lipoproteins, quenches singlet oxygen, and boosts the immune system (Zhu et al. 2009). Animals are unable to synthesize carotenoids directly and must obtain them from their diets. β-Carotene and astaxanthin are typical ingredients for chicken and fish feeding, respectively. Although fruits and vegetables are particularly good sources of certain carotenoids, cereal grains generally lack these compounds, leading to deficiency diseases in countries where cereals are the staple diet (Zhu et al. 2007; Bai et al. 2011; Berman et al. 2015). Also, with the exception of *Adonis aestivalis* flowers, astaxanthin and other ketocarotenoids are scarce in plants, although they are abundant in fish and shellfish and are used in aquaculture to enhance the aesthetic qualities of salmon (Zhu et al. 2009). For these reasons, there is much interest in modifying cereal crops, such as maize and rice, to enhance the carotenoid content (Zhu et al. 2007, 2008, 2009; Naqvi et al. 2011a; Bai et al. 2011; Farre et al. 2010, 2011, 2012, 2014, 2015; Berman et al. 2015).

8.2 Combinatorial Nuclear Transformation Generates a Diverse Library of Plants with Distinct and Stable Phenotypes

We used as a model system the South African elite white maize inbred M37W, which lacks carotenoids in the endosperm due to the absence of the enzyme phytoene synthase (PSY1). We transformed 13-day-old immature zygotic embryos by bombarding them with gold particles coated with six constructs, the selectable marker *bar*, and five carotenogenic genes: *Zmpsy1* (*Zea mays* phytoene synthase 1), *PacrtI* (*Pantoea ananatis* phytoene desaturase), *Gllycb* (*Gentiana lutea* lycopene β-cyclase), *Glbch* (*G. lutea* β-carotene hydroxylase, a plant-type β-ring non-heme di-iron monooxygenase introducing hydroxy groups at C-3), and *ParacrtW* (*Paracoccus* β-carotene ketolase) (Zhu et al. 2008). Each gene was driven by a different endosperm-specific promoter (respectively, the wheat low-molecular-weight glutenin, barley D-hordein, rice prolamin, rice glutelin-1, and

maize γ-zein). A population of regenerated plants was screened by genomic PCR, revealing many different combinations of transgenes, including nine lines (13%) containing all five carotenoid input transgenes. Multiple independent transgenic lines containing and expressing the same transgene complement were identified. All of the transgenic plants showed normal morphology and development, reflecting the restriction of transgene expression to the seed endosperm. Visual inspection of the endosperm tissue revealed seven distinct phenotypes based on endosperm color (Ph-1 to Ph-7; Fig. 8.1a). Analysis of steady-state mRNA levels revealed which transgenes were expressed in individual transgenic plants (Fig. 8.1b). We found a precise correlation between the phenotypes and expressed transgenes. Phenotype 1 (Ph-1), expressing *Zmpsy1* alone, appeared similar in color to WT yellow maize, whereas Ph-2, expressing *PacrtI* alone, was very pale yellow in color. The combination of *Zmpsy1* and *PacrtI* in Ph-3 generated an orange-red phenotype, whereas the combination of *Gllycb* in addition to *Zmpsy1* and *PacrtI* in Ph-4 produced a distinct orange-yellow color. Ph-5, Ph-6, and Ph-7 were more complex phenotypes resulting from the expression of a bacterial ketolase gene, *ParacrtW*, in addition to *Zmpsy1* + *PacrtI* + *Glbch*, *Zmpsy1* + *PacrtI* + *Gllycb*, or *Zmpsy1* + *PacrtI* + *Gllycb* + *Glbch*, respectively. These lines also had distinguishable orange to red phenotypes (Fig. 8.1a).

8.3 Reconstruction of the Carotenoid Pathway in White Maize Leads to the Accumulation of Extraordinary Levels of Metabolic Intermediates and End Products

HPLC analysis showed that the different color phenotypes reflected the accumulation of different metabolites (Fig. 8.1a and Table 8.1), confirming a direct correspondence between genotype and carotenoid accumulation. The metabolic profiles of each transgene complement and resulting phenotype were qualitatively consistent in the multiple transgenic events tested, despite the variation in the absolute levels of particular compounds. Ph-1 (*Zmpsy1* alone) showed a 53-fold increase in the total carotenoids over white maize [58.21 vs. 1.10 μg/g dry weight (DW)], whereas Ph-2 (*PacrtI* alone) and Ph-3 (*Zmpsy1* + *PacrtI*) showed 2.5- and 142-fold increases, respectively (2.69 and 156.14 μg/g DW) (Table 8.1). The total carotenoid content in Ph-4 (*Zmpsy1* + *PacrtI* + *Gllycb*) reached 148.78 μg/g DW. These data confirm that PSY1 is the key enzyme-limiting carotenoid accumulation in the endosperm of white maize. The predominant carotenoids accumulating in Ph-1 endosperm were zeaxanthin (18.25 μg/g DW, 31.35% of the total carotenoids), lutein (14.95 μg/g DW, 25.68%), and β-carotene (7.10 μg/g DW, 12.20%), whereas those accumulating in Ph-3 were β-carotene (57.35 μg/g DW, 36.73%) and lycopene (26.69 μg/g DW, 17.09%). These findings suggest that both lycopene cyclases may be rate-limiting in the synthesis of cyclic carotenes in Ph-3.

Simultaneous expression of *Zmpsy1*, *PacrtI*, and *Gllycb* in Ph-4 dramatically reduced the levels of lycopene (11.50 μg/g DW, 7.73%) but increased the levels of zeaxanthin (34.53 μg/g DW, 23.21%) compared with Ph-3 (Table 8.1). Phytoene, which is not present in WT M37W endosperm, accumulated in Ph-1, Ph-3, and Ph-4 (which express *Zmpsy1*), but not in Ph-2 (which does not express *Zmpsy1*) (Table 8.1). This finding indicates that the conversion of phytoene to lycopene is a subsequent limiting step for carotenoid biosynthesis. Phenotypes and carotenoid content remained stable for nine generations (homozygous T9 plants) (Zanga et al. 2016). Multiple independent transgenic plants expressing the same transgene complement exhibited identical qualitative metabolite profiles.

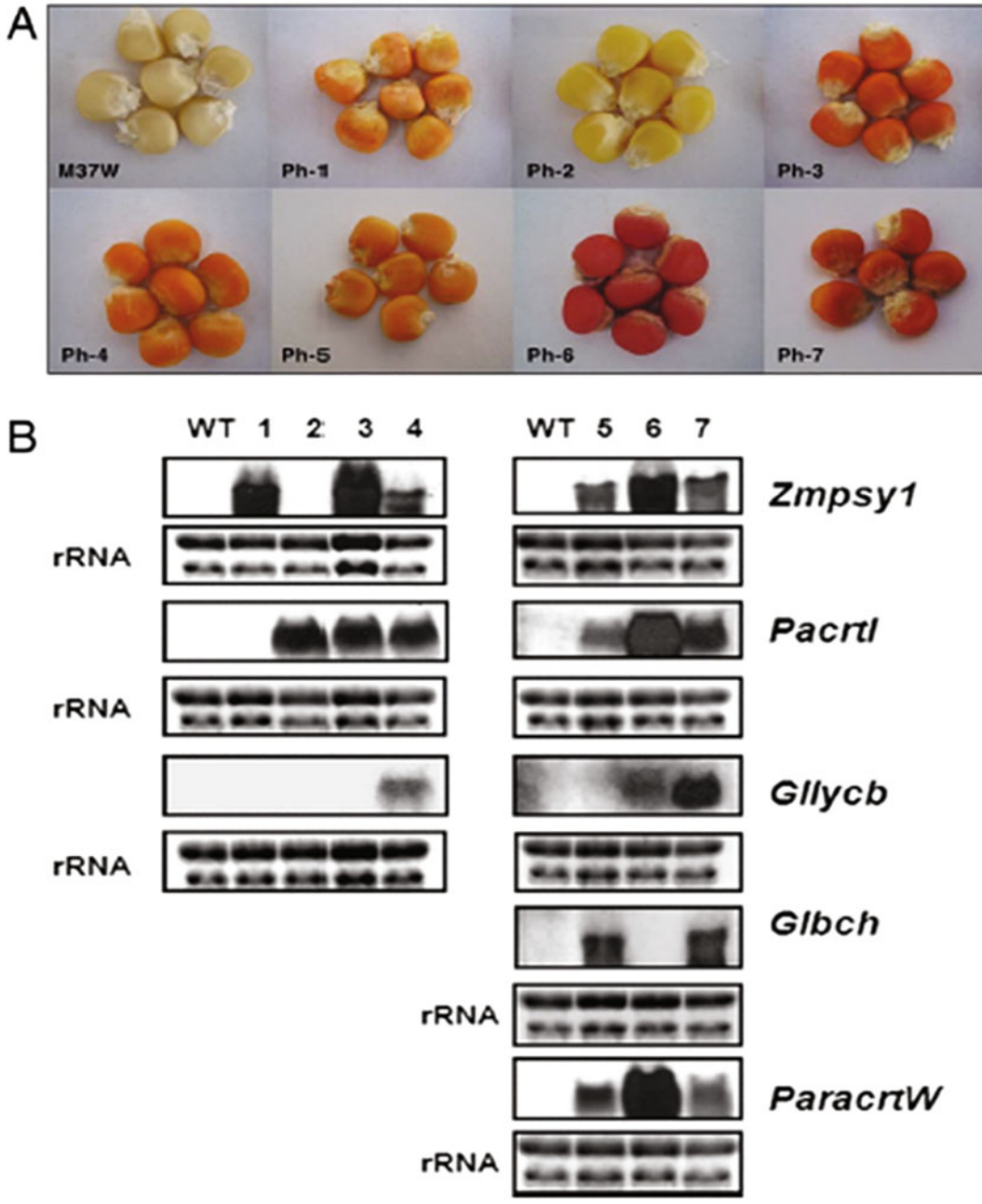

Fig. 8.1 Phenotypes and genotypes of seven combinatorial transformants (Zhu et al. 2008). (**a**) Endosperm colors of seven different transgenic maize phenotypes. Ph-1 expressing *Zmpsy1* only accumulates zeaxanthin and has a bright yellow color. Ph-2 expressing only *PacrtI* has a phenotype similar to WT M37W with a slight increase in total carotenoids. Ph-3 (*Zmpsy1* and *PacrtI*) accumulates a significant amount of β-carotene and lycopene and has an orange-red color. Ph-4 expresses *Gllycb* in addition to *Zmpsy1* and *PacrtI* and accumulates β-carotene, hence the orange color. Ph-5, Ph-6, and Ph-7 express *Zmpsy1* + *PacrtI* + *Glbch*, *Zmpsy1* + *PacrtI* + *Gllycb*, and *Zmpsy1* + *PacrtI* + *Gllycb* + *Glbch*, respectively, in addition to *ParacrtW*, thus showing a range of colors from orange to red, depending on the accumulation of ketocarotenoids. (**b**) Northern blot analysis (30 μg of total RNA per lane) to monitor transgene expression in WT M37W and the seven transgenic phenotypes (lanes 1–7). Staining of rRNA with ethidium bromide was used as a loading control

Table 8.1 Carotenoid content and composition in white maize (M37W) and Ph-1 to Ph-7 (Zhu et al. 2008)

Content	M37W	Ph-1	Ph-2	Ph-3	Ph-4	Ph-5	Ph-6	Ph-7
Phytoene*	0	10.81 ± 2.79	0	9.40 ± 2.42	10.68 ± 3.16	17.53 ± 2.72	18.93 ± 3.86	15.79 ± 2.43
Lycopene	0	0	0	26.69 ± 10.65	11.50 ± 3.87	2.68 ± 0.94	12.61 ± 1.49	2.38 ± 0.97
γ-Car	0.07 ± 0.03	0	0	3.31 ± 0.19	3.81 ± 0.28	1.45 ± 0.36	1.55 ± 0.14	1.24 ± 0.50
α-Car	0.09 ± 0.02	0.93 ± 0.08	0	6.10 ± 0.65	6.60 ± 0.32	1.58 ± 0.47	5.56 ± 1.28	2.48 ± 0.93
β-Car	0.14 ± 0.05	7.10 ± 1.45	0	57.35 ± 5.77	48.87 ± 6.83	8.72 ± 2.41	34.81± 4.37	25.78 ± 2.46
α-Cryptox	0	3.68 ± 0.86	0	12.2 ± 2.32	11.33 ± 2.48	5.29 ± 0.95	12.94 ± 1.80	2.45 ± 0.82
β-Cryptox	0	2.49 ± 0.75	0	5.97 ± 0.65	9.34 ± 0.39	3.38 ± 0.89	1.85 ± 0.38	4.95 ± 0.29
Lutein**	0.53 ± 0.14	14.95 ± 2.54	1.57 ± 0.98	9.76 ± 1.32	13.12 ± 1.40	18.11 ± 2.68	9.14 ± 1.08	12.27 ± 0.73
Zeaxan	0.27 ± 0.08	18.25 ± 1.98	1.12 ± 0.64	25.36 ± 3.42	34.53 ± 2.89	27.47 ± 3.92	13.71± 1.81	16.78 ± 2.94
3-HO-Echin						0	3.77 ± 0.85	3.64 ± 0.69
Echin						0	5.06 ± 0.48	1.86 ± 0.28
Adonix						10.62 ± 2.69	22.36 ± 3.52	12.48 ± 1.46
Astax						0	4.46 ± 1.29	0
Total carot	**1.10**	**58.21**	**2.69**	**156.14**	**148.78**	**96.83**	**146.75**	**102.10**
β/ε ratio	0.77	1.42	0.71	3.28	3.11	2.07	3.17	3.88
% Ketoderiv						10.96	24.29	17.61

Abbreviations: *Ph* Phenotype, *γ-Car* γ-Carotene, *α-Car* α-Carotene, *β-Car* β-Carotene, *α-Cryptox* α-Cryptoxanthin, *β-Cryptox* β-Cryptoxanthin, *Zeaxan* Zeaxanthin, *3-HO-Echin* 3-hydroxy-Echinenone, *Echin* Echinenone, *Adonix* Adonixanthin, *Astax* Astaxanthin, *β/ε ratio* the ratio of β-ring to ε-ring derivatives, *% Ketoderiv* % Ketoderivatives, including *phytoene epoxide and **lutein epoxide; Value presented in μg/g DW; ± standard deviation of 3 to 5 individual T2 mature seeds

8.4 Reconstruction of the Astaxanthin Biosynthesis Pathway in Maize Endosperm Reveals a Metabolic Bottleneck in the Conversion of Adonixanthin Into Astaxanthin by β-carotene Ketolase

Astaxanthin is formed from β-carotene by the addition of keto groups at the 4 and 4′ positions and hydroxyl groups at the 3 and 3′ positions of the β-ionone rings. These reactions are catalyzed by β-carotene ketolase and β-carotene hydroxylase, respectively (Zhu et al. 2009). Ph-4 endosperm (*Zmpsy1* + *PacrtI* + *Gllycb*) accumulated not only β-carotene but also xanthophylls, such as lutein and zeaxanthin. The pathway can be extended further to ketocarotenoids, such as astaxanthin, by expressing *ParacrtW*. We generated three unique phenotypes in which the *ParacrtW* transgene was expressed in combination with *Zmpsy1* and *PacrtI*, differing in the additional expression of *Glbch* (Ph-5), *Gllycb* (Ph-6), or both *Glbch* and *Gllycb* (Ph-7, which expressed all five carotenogenic input transgenes) (Fig. 8.1b). In terms of ketocarotenoid synthesis, HPLC analysis revealed the presence of adonixanthin (4-ketozeaxanthin) in Ph-5, adonixanthin, echinenone (4-keto-β-carotene), and 3-hydroxyechinenone in Ph-7 and these three carotenoids plus astaxanthin (3,3′-dihydroxy-4,4′-diketo-β-carotene) in Ph-6 (Table 8.1). The predominant carotenoids accumulating in Ph-5 were zeaxanthin (27.47 μg/g DW; 28.37% of total carotenoids) and lutein (18.11 μg/g DW; 18.71%) in addition to ketocarotenoids (10.62 μg/g DW; 10.96%) (Table 8.1). Ph-5 is "based on" Ph-3, having the Ph-3 genotype with additional genes *Glbch* and *ParacrtW*. Ph-5 accumulated less β-carotene than Ph-3 (8.72 vs. 57.35 μg/g DW), probably reflecting the conversion of β-carotene into β-cryptoxanthin and zeaxanthin by GlBCH (Table 8.1). Similarly, Ph-7 is based on Ph-4, having the Ph-4 genotype with additional genes *Glbch* and *ParacrtW* (Fig. 8.1b), and it produced mainly β-carotene (25.78 μg/g DW; 25.24%) and zeaxanthin (16.78 μg/g DW; 16.43%) in addition to ketocarotenoids (17.98 μg/g DW; 17.61%) (Table 8.1). Ph-6 (*Zmpsy1* + *PacrtI* *Gllycb* + *ParacrtW*) accumulated significant amounts of ketocarotenoids (35.85 μg/g DW; 24.29%) and β-carotene (34.81 μg/g DW; 23.72%) but less zeaxanthin (13.71 μg/g DW; 9.34%), most likely because of the absence of GlBCH (Fig. 8.1b). Ph-7 accumulated the three mono-ketocarotenoids echinenone, 3-hydroxy-echinenone, and adonixanthin, whereas Ph-5 produced only adonixanthin. The missing echinenone and 3-hydroxy-echineneone in Ph-5 may be caused by a shortage of β-carotene (8.72 μg/g DW), reflecting the absence of lycopene β-cyclase (Fig. 8.1b). In contrast, Ph-6 produced not only the three mono-ketocarotenoids but also the di-ketocarotenoid astaxanthin (Table 8.1). The astaxanthin yield in Ph-6, however, is considerably lower than β-carotene in Ph-3. The limited astaxanthin synthesis is due to a restricted conversion of adonixanthin (3,3′-dihydroxy-4-keto-β-carotene, 4-keto zeaxanthin), resulting in the major accumulation of adonixanthin among ketocarotenoids in Ph-5 (100% adonixanthin/total ketocarotenoids), Ph-6 (72%), and Ph-7 (69%) (Table 8.1).

Phytoene was not detected in WT M37W endosperm, but it accumulated in all of the transgenic varieties with the exception of Ph-2 (the only one lacking *Zmpsy1*). This suggests that the conversion of phytoene to lycopene (catalyzed by endogenous desaturases and isomerases in Ph-1 and by PaCRTI in addition to endogenous desaturases and isomerase in the other phenotypes) is a rate-limiting step for carotenoid biosynthesis in these phenotypes. The expression of *Zmpsy1* and *PacrtI* genes in Ph-3 led to the accumulation of lycopene (Table 8.1), the product of the enhanced phytoene synthase and desaturase branch of the pathway, suggesting that lycopene cyclases are rate-limiting steps in the conversion of lycopene to cyclic carotenes in phenotype-3. The addition of *G. lutea* lycopene β-cyclase (*Gllycb*) gene in Ph-4 alleviated this partial pathway limitation by converting lycopene

preferentially to β-carotene and further derivatives (Table 8.1).

Our approach provides a unique and surprisingly straightforward strategy for metabolic pathway analysis and multigene metabolic engineering in plants. It involves the introduction and coordinated expression of multiple transgenes followed by the selection of stable lines expressing the specific combination of transgenes required for particular metabolic outputs. Individual lines, producing specific metabolites (Ph-3 for β-carotene and lycopene; Ph-4 for β-carotene and zeaxanthin; and Ph-6 for astaxanthin), can be goals in themselves if the aim is to accumulate particular molecules. However, by examining the entire diverse population of plants, it becomes possible to dissect the pathway and subsequently reconstruct it either in its original form or with modifications, thus providing a basis for understanding and subsequently engineering novel metabolites. The broad significance of this approach is that it considerably simplifies the process of carotenoid metabolic engineering by making it analogous to screening a library of metabolic variants for the correct functional combination. This approach could be applied to any pathway given a suitable template for combinatorial transformation.

8.5 Synergistic Metabolism in Hybrid Corn Indicates Bottlenecks in the Carotenoid Pathway and Leads to the Accumulation of Extraordinary Levels of the Nutritionally Important Carotenoid Zeaxanthin

Lutein and zeaxanthin cannot be synthesized de novo in humans, and although lutein is abundant in fruit and vegetables, good dietary sources of zeaxanthin are scarce. Transgenic line Ph-4 expressing *Zmpsy1*, bacterial *PacrtI*, and *Gllycb* in a M37W background was used to introgress the transgenic mini-pathway into the yellow endosperm EP42 and A632 backgrounds (Naqvi et al. 2011b). The novel hybrids, designated as Ph-4 x EP42 and Ph-4 x A632, were selfed for two generations to obtain homozygous lines expressing all three transgenes. RT-PCR analysis was then carried out on endosperm tissue from wild-type M37W, EP42, and A632 plants along with Ph-4, Ph-4 x EP42, and Ph-4 x A632. The three transgenes were expressed as strongly in the hybrid lines as in the transgenic parent (Naqvi et al. 2011b). The hybrid lines had a bright orange endosperm phenotype, indicating the accumulation of more carotenoids, or different carotenoids compared with the transgenic parent (Fig. 8.2a). HPLC analysis showed that the wild-type M37W endosperm contained a maximum of 1.42 μg/g DW total carotenoids, which increased to a maximum of 127 μg/g DW in transgenic line Ph-4 where it comprised up to 10.42 μg/g DW lycopene, 41.20 μg/g DW β-carotene, and 29.64 μg/g DW zeaxanthin (Fig. 8.2b). Ph-4 also accumulated phytoene and other intermediates, such as α- and β-cryptoxanthin and α-carotene at lower levels (Naqvi et al. 2011b). In Ph-4 x EP42, the total carotenoid content increased to 90.32 μg/g DW, including 19.31 μg/g DW β-carotene, 23.41 μg/g DW lutein, and 38.07 μg/g DW zeaxanthin. In Ph-4 x A632, the total carotenoid content was 88.53 μg/g DW, including 15.24 μg/g DW β-carotene, 9.72 μg/g DW lutein, and 56.49 μg/g DW zeaxanthin (Table 8.2). Both lines also accumulated intermediates, such as α- and β-cryptoxanthin, but unlike the Ph-4 parent, there was no evidence of phytoene or lycopene. The β:ε ratio of both hybrids was significantly higher than the wild-type parents, 2.05 in the case of Ph-4 x EP42, and 6.80 in the case of Ph-4 x A632. These results demonstrate that introgressing the transgenic mini-pathway into wild-type yellow endosperm varieties gives rise to hybrids in which the β:ε ratio is altered additively. Where the β:ε ratio in the genetic background is high, introgression of the mini-pathway allows zeaxanthin production at an unprecedented 56 μg/g DW. This result shows that metabolic synergy between endogenous and heterologous pathways can be used to enhance the levels of nutritionally important metabolites.

As shown in Fig. 8.2b and Table 8.2, Ph-4 accumulated not only high levels of zeaxanthin

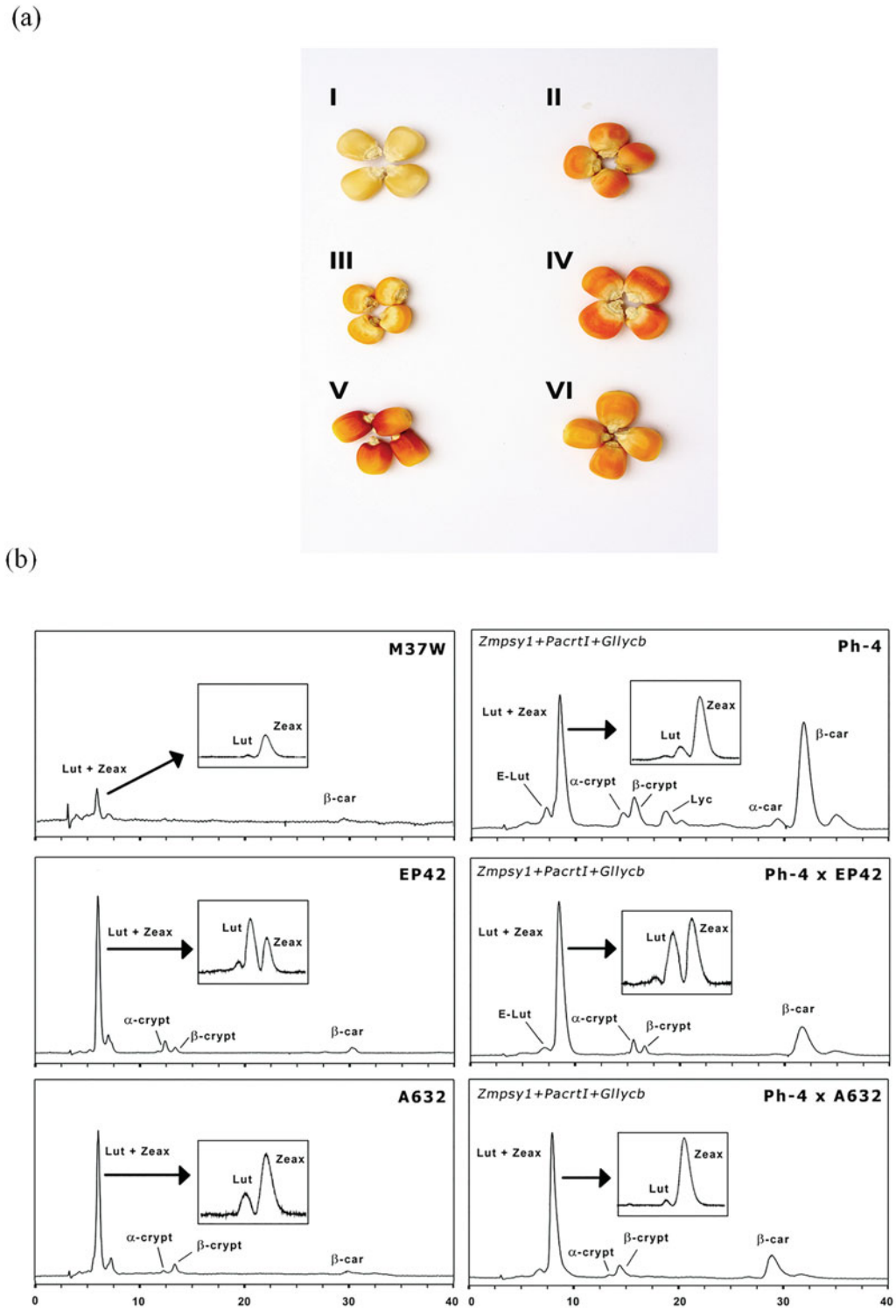

Fig. 8.2 (**a**) Color phenotype shows carotenoid accumulation in endosperm (Naqvi et al. 2011b): (I) M37W; (II) Ph-4; (III) EP42; (IV) Ph-4 x EP42; (V) A632; and (VI) Ph-4 x A632. (**b**) HPLC analysis of carotenoids in wild-type (M37W, EP42, and A632), transgenic (Ph-4), and hybrid (Ph-4 x EP42 and Ph-4 x A632) corn lines, with retention time shown on

and lutein but also carotenoid intermediates, such as phytoene, lycopene, α- and β-cryptoxanthin, and α- and β-carotene. In the hybrid lines Ph-4 x A632 and Ph-4 x EP42, however, these intermediates were not present, suggesting that additional, more subtle bottlenecks in the M37W genetic background were also alleviated by metabolic complementation. M37W endosperm accumulates only traces of carotenoids because of the lack of *psy1* expression. In contrast, *psy1* mRNA is abundant in both yellow corn inbreds (EP42 and A632) (Naqvi et al. 2011b). The traces of lutein and zeaxanthin in M37W endosperm probably reflect the presence of the *psy2* transcript, which is mainly responsible for carotenoid biosynthesis in green tissues but may have some residual activity in the endosperm. Interestingly, Ph-4 contains a significant amount of phytoene (7.36 μg/g DW), which suggests that the next step in the pathway (the conversion of phytoene into lycopene by the bacterial enzyme phytoene desaturase) is limiting. In contrast, no phytoene was detected in yellow corn nor in the hybrids, demonstrating that the three enzymes carrying out the corresponding endogenous reactions in yellow corn are not limiting and alleviate the bottleneck in the M37W background when the induced and endogenous pathways are combined in the hybrid. Similarly, the transgenic endosperm also contained significant amounts of lycopene (10.42 μg/g DW), whereas no lycopene was detected in either hybrid (Table 8.2). Phytoene and lycopene accounted for ca: 14% of total carotenoids in Ph4. Again, this suggests that the lycopene β-cyclase provided by the transgene introduces a bottleneck in the M37W background, which is overcome by the additional lycopene β-cyclase activity in yellow corn. The disappearance of carotene intermediates, such as phytoene and lycopene, in the two hybrids might also be due, at least in part, to lower plastidial methyl erythritol 4-phosphate pathway-derived isoprenoid precursor availability in the yellow endosperm lines and/or the higher catabolic activities of zeaxanthin epoxidase and carotenoid cleavage dioxygenase (CCDs) in the yellow endosperm backgrounds. Both hybrid lines contained significant amounts of β-carotene (19 μg/g DW in Ph-4x EP42 and 15 μg/g DW in Ph-4 x A632), but this was much lower than the 41 μg/g DW we measured in the transgenic parent. A plausible reason for this difference might be the higher levels of *bch2* accumulation in the two yellow lines compared with M37W (Naqvi et al. 2011b).

It is unlikely that differences in lutein accumulation between EP42 and A632 (and the corresponding hybrids with Ph-4) are due to cytochrome p450-type hydroxylases, as expression levels of these genes, at least at the mRNA level, were very similar (Naqvi et al. 2011b). These data suggest that the yellow corn backgrounds also alleviated a bottleneck in β-carotene hydroxylase activity, allowing the efficient flow of intermediates towards zeaxanthin synthesis. Our collective data indicate that the yellow corn background compensated for inefficient activity at every step of the pathway conferred by the transgenes, but the combination of reduced lycopene ε-cyclase activity and the pooled lycopene β-cyclase activity in the hybrid conferred its highly skewed β:ε ratio and its extraordinary potential to accumulate zeaxanthin. This study is the first to show that significant increases in zeaxanthin levels in a food crop can be achieved by combining conventional breeding with genetic engineering. Whereas genetic engineering provides advantages such as speed and access beyond the species gene pool, it can be difficult and/or time-consuming to transform

Fig. 8.2 (continued) the x-axis and intensity on the y-axis (Naqvi et al. 2011b). Zeaxanthin and lutein were separated in parallel runs using a C18 Vydac 218TP54 column, with methanol containing 2% water as the mobile phase. Samples were monitored with a Kontron DAD 440 photodiode array detector with online registration of the spectra. Abbreviations: *E-lut* epoxylutein, *Lut* lutein, *Zeax* zeaxanthin, *β-car* β-carotene, *α-crypt* α-cryptoxanthin, *β-crypt* β-cryptoxanthin, *lyc* lycopene, and *α-car* α-carotene

Table 8.2 Carotenoid composition and content of wild-type (M37W, EP42, and A632), transgenic (Ph-4), and hybrid (Ph-4 and Ph-4 x A632) corn endosperm (Naqvi et al. 2011b) (presented as μg/gDW ± SD) (n = 3–5 mature T_3 seeds)

Plants	Phy	Lyc	γ-Car	α-Car	β-Car	α-Crypt	β-Crypt	Lut	Zeax	CAR	β/α ratio
M37W	0	0	0	0	0	0	0	0.64 ± 0.21	0.78 ± 0.30	1.42	1.21
EP42	0	0	0	0	1.41 ± 0.75	2.39 ± 0.60	1.94 ± 0.86	15.88 ± 1.72	7.92 ± 1.34	29.54	0.61
A632	0	0	0	0	1.29 ± 0.31	1.43 ± 0.45	1.50 ± 0.27	7.69 ± 2.31	14.53 ± 2.90	26.42	1.90
Ph4	7.36 ± 2.30	10.42 ± 1.95	3.30 ± 0.56	5.87 ± 0.94	41.20 ± 3.21	7.65 ± 1.37	11.28 ± 1.83	10.76 ± 2.47	29.64 ± 2.41	127.48	3.51
Ph4xEP42	0	0	0	0.72 ± 0.31	19.31 ± 2.48	5.46 ± 1.82	3.35 ± 0.92	23.41 ± 3.56	38.07 ± 4.32	90.32	2.05
Ph4xA632	0	0	0	0	15.24 ± 2.63	1.62 ± 0.51	5.46 ± 1.49	9.72 ± 1.82	56.49 ± 3.19	88.53	6.80

Abbreviations: *Phy* phytoene, *Lyc* lycopene, *γ-Car* γ-carotene, *α-Car* α-carotene, *α-Crypt* α-cryptoxanthin, *β-Crypt* β-cryptoxanthin, *Lut* lutein and epoxylutein, *Zeax* zeaxanthin, *CAR* total carotenoids, *β/α ratio* the ratio of β, *β-carotenoids* (γ-carotene, β-carotene, and β-cryptoxanthin and zeaxanthin) to β, *ε-carotenoids* (δ-carotene, α-carotene, and α-cryptoxanthin and lutein)

locally adapted varieties directly and therefore make a practical impact on nutrition and health, particularly in developing countries where staples such as corn represent the predominant food source for many people. Conventional breeding for improved nutrition is slow and laborious, particularly where the intent is to modify several different metabolic pathways simultaneously (Naqvi et al. 2009), and is limited to the gene pools of compatible species. Our combined approach cherry-picks the advantages of both systems—the speed, power, and accessibility of genetic engineering and the diversity and practicality of conventional breeding—to generate nutritionally enhanced crops with unprecedented levels of a key nutrient in the human diet.

8.6 Combined Transcript, Proteome, and Metabolite Analysis of Transgenic Maize Seeds Engineered for Enhanced Carotenoid Synthesis Reveals Pleiotropic Effects in Core Metabolism

Transcriptomic, proteomic, and metabolomics/metabolite profiling are highly useful for detecting metabolic changes in transgenic plants (Ricroch et al. 2011). Genetic engineering of a pathway for the higher accumulation of an end product may have a global effect on the whole metabolism (Sandmann 2001). Increased precursor utilization can negatively affect closely related pathways competing for the same precursors. The engineered maize line Ph-3 described earlier was subjected to an in-depth metabolomics, transcriptomics, and proteomics analysis and compared with its near-isogenic line M37W (Decourcelle et al. 2015). The aim of this study was to evaluate whether endosperm-specific carotenoid biosynthesis influenced core metabolic processes in maize embryo and endosperm and how global seed metabolism adapted to this expanded biosynthetic capacity.

Carotenoid composition and the concentrations of other terpenoids, sterols, and tocopherol were analyzed in the endosperm and embryo. More than 90% of the carotenoids in the non-transgenic variety M37W are located in the embryo, mainly zeaxanthin and violaxanthin. However, Ph-3 had a 20-fold increase in carotenoid composition, mostly zeaxanthin and β-cryptoxanthin. This increase in carotenoid synthesis in the endosperm operates, at least in part, at the expense of the synthesis in the embryo, which was only half of that found in the non-transgenic line. Another change in the embryo carotenoid composition was the amount of lutein and α-cryptoxanthin in Ph-3. γ-Tocopherol was found exclusively in the embryo which demonstrated a precise separation of both compartments. Sterol content was also threefold higher in the embryo when compared with the endosperm, and the major sterols identified in higher concentrations were stigmasterol and sitosterol. The only difference between M37W and the transgenic line was an increase in the sitosterol content in the embryo of Ph-3. Sucrose and sorbitol concentrations increased and glucose, fructose, and xylose concentrations decreased in Ph-3. Other metabolites from early glycolysis with lower concentrations in Ph-3 were glycolate and glycerate. In addition, aspartate and proline concentrations increased in Ph-3. Palmitic, stearic, and oleic acids were detected with an increase that is greater than eightfold. The metabolomic data indicate a higher rate of fatty acid synthesis which should also occur in the embryo. Unexpectedly, sterol and fatty acid syntheses were also higher in the transgenic line. We thus demonstrated that the transgenic line needed a higher flux through the glycolytic pathway for the synthesis of carotenoids, sterols, and fatty acids (Decourcelle et al. 2015).

8.7 Metabolic Engineering of Ketocarotenoid Biosynthesis in Maize Endosperm and Characterization of a Prototype High Oil Astaxanthin-Enriched Hybrid

The white endosperm M37W inbred was transformed with β-carotene hydroxylase (*crtZ*)

from *Brevundimonas* and β-carotene ketolase from *Chlamydomonas reinhardtii* to extend the carotenoid pathway to astaxanthin. In addition, phytoene synthase 1 was also overexpressed, and lycopene ε-cyclase was knocked down to direct the pathway towards the β-branch in line bkt. After crossing the astaxanthin pathway in bkt into the high-oil NSL76 line, astaxanthin accumulation in the hybrid seeds increased by ca: 50% compared with the original astaxanthin accumulating bkt line. The NSL76-bkt hybrid line accumulated 60% of the total seed carotenoids as astaxanthin (Farre et al. 2016).

NSL-bkt and its parental line bkt were used for metabolomic and proteomic analysis. The enhanced metabolite flow into the carotenoid pathway affected primary metabolism as a source for terpenoid pathway precursors. Sucrose and lactate pools were increased in the endosperm. The concentration of sucrose synthase was decreased, providing less UDP glucose for the synthesis of other sugars, such as trehalose. These changes in enzyme concentrations preferentially supported glycolytic metabolism to the precursors for carotenoid biosynthesis. Lactate accumulation coincided with lower activity pools of the citric acid cycle components and lower concentrations of malate dehydrogenase. This indicates a reduced flow of pyruvate into the citric acid cycle. It appears that pathways competing with glycolytic pyruvate formation or competing with the deoxyxylulose 5-phosphate pathway for pyruvate were downregulated. The amino acid pool was decreased in NSL76-bkt, and the concentrations of a legumin-like protein and glutamin-enriched storage protein were increased/decreased, respectively (Farre et al. 2016).

8.8 Carotenoid-Enriched Transgenic Corn Delivers Bioavailable Carotenoids to Poultry and Protects Them Against Coccidiosis

Vitamin A and carotenoid metabolism in poultry is closely related to the equivalent process in humans, so chickens are also susceptible to vitamin A deficiency with similar symptoms as humans (Pretorius and Schönfeldt 2013). High-carotenoid (HC) corn was used in poultry feeding experiments to ascertain its efficiency to maintaining poultry productivity and health (Nogareda et al. 2016).

The typical corn- and soybean-based commercial poultry diets do not supply sufficient carotenoids to produce the golden skin preferred by many consumers and do not confer additional health benefits. Vitamin A and natural or synthetic pigments are routinely added to poultry feed, increasing the production costs (Castaneda et al. 2005).

We investigated the potential use of carotenoid-enriched transgenic corn in several chicken trials using commercial broilers (Ross 308 males). Birds were fed on diets supplemented with 58% of HC corn and were compared with a nutritionally equivalent diet supplemented with its near-isogenic line, M37W, which is essentially devoid of carotenoids. Chickens were raised under controlled experimental conditions at the University of Lleida research animal facilities. The birds were slaughtered at normal weight for commercial production and subjected to gross necropsy, histopathology, and blood chemistry, and selected tissues and organs were analyzed for carotenoid accumulation.

No differences in the growth, final body weight, histopathology, blood chemistry, or the final weight of most organs (liver, heart, and spleen) were observed with the exception of the bursa of Fabricius in replicated trials (Nogareda et al. 2016). The bursa of Fabricius is a lymphoid gland located on the posterodorsal wall of the cloaca that regresses with sexual maturity and plays an important role in disease resistance. This organ was heavier in the birds fed on the HC-supplemented diet. Histopathological analysis of bursa of Fabricius showed no evidence of any healthy problem, and we concluded that the higher weight of this organ in animals fed on the HC-supplemented diet may have been caused by a better immunomodulation to a previous vaccination.

The analysis of hemoglobin levels, hematocrit values, and ratio of different blood cell types showed no differences between the diet groups. In both cases, the values were similar to standard chicken references (Fudge 2000; Harrison and Lightfoot 2006).

The CIELAB trichromatic system was used to quantify the lightness, redness, and yellowness of pre-chilled meat and skin tissue from birds in both diet groups (Nogareda et al. 2016). This revealed significant differences ($P < 0.001$) in the skin, meat color, and external cutaneous structures, such as the comb and base of the feathers. Chickens raised on the HC-supplemented diet were healthy and accumulated higher levels of bioavailable carotenoids in peripheral tissues, muscle, skin, and fat. The analysis of carotenoid levels in breast meat showed that violaxanthin and β-cryptoxanthin were only present in the birds fed on the HC-supplemented diet and that the levels of lutein, zeaxanthin, and β-carotene were significantly higher ($P < 0.001$) in the breast meat of birds fed on the HC-supplemented diet. We found that the levels of lutein, zeaxanthin, and β-carotene were 117% (2-fold), 2104% (22-fold), and 999% (11-fold) higher in birds reared on the HC-supplemented diet. In contrast, violaxanthin and β-cryptoxanthin were not present in thigh meat, lutein levels were similar in both diet groups, zeaxanthin levels were significantly higher ($P < 0.001$) in the thigh meat of birds fed on the HC-supplemented diet, and there was no β-carotene in the thigh meat, suggesting that it had been metabolized into downstream derivatives. Accordingly, the thigh muscle of the birds fed on the HC-supplemented diet contained substantial amounts of oxidation products, such as zeaxanthin-5,8-epoxides and β-carotene-5,8-epoxides, suggesting that high levels of zeaxanthin and β-carotene may have accumulated initially but subsequently underwent oxidation (Nogareda et al. 2016).

The livers of birds reared on the HC-supplemented diet accumulated high levels of retinol and also a retinoic acid conjugate that was not present in the livers of control animals. Retinol in the liver was almost double compared with the control diet (814 ± 115 and 471 ± 52 μg/g freeze-dried liver, respectively) (Nogareda et al. 2016). Vitamin A metabolism is complex and involves many different biologically active molecules (retinol, retinal, retinoic acid, and oxidized and conjugated metabolites) which are collectively known as retinoids (D'Ambrosio et al., 2011). The higher levels of serum and liver retinoids in birds fed on the HC-supplemented diet are likely to be derived from the tenfold higher supply of provitamin A carotenoids present in the feed. The absence of β-carotene in most of the tissues we tested, coupled with the higher levels of β-carotene oxidation products in the skin and muscle and the higher levels of retinol in the liver and serum, suggests that β-carotene obtained from the diet is metabolized to retinol via retinal or oxidized in line with its antioxidant activity. A similar trial was conducted under farm conditions. The results were similar to those we obtained under the controlled experimental conditions.

Having confirmed the nutritional and color-promoting activity of HC in enriched poultry diets, we carried out an additional trial incorporating a challenge with *Eimeria tenella*, one of several important protozoan parasites that cause coccidiosis, which is an important disease in commercial broilers farms (Nogareda et al. 2016). We allocated 56 1-day-old chicks into four groups of 14 animals housed in isolated cages. Two groups of chickens were fed on the HC-supplemented diet and two on the control diet. One group from each diet was challenged orally with an *E. tenella* inoculum of 24.3×10^4 sporulated oocysts (Houghton strain) on day 13. Cecal intestinal lesions, footpad dermatitis, digital ulcers, and fecal oocyst counts post-challenge were evaluated. Animals in the challenged groups weighed 25% less than their counterparts in the non-challenged groups 6 days post-challenge. Cecal intestinal lesions were assigned a score from 0 to 4, depending on the severity. The challenged group on the HC-supplemented diet had fewer lesions than the controls (the average scores of the animals were 0 in the non-challenged groups, 2.75 ± 0.41 in the challenged group on the HC-supplemented diet, and 3.25 ± 0.16 in the control diet). Birds on the

HC-supplemented diet suffered substantially milder disease symptoms and had lower fecal oocyst counts than birds on the control diet. The number of oocysts per gram of feces was 0 in the non-challenged groups, and significantly fewer oocysts were found ($P < 0.05$) in the feces of challenged chickens fed on the HC-supplemented diet on day 6 post-challenge (87,000 ± 1200 and 132,800 ± 2900 oocysts/g feces) and day 9 post-challenge (14,200 ± 900 and 56,900 ± 4300 oocysts/g feces) compared with challenged control diets (Nogareda et al. 2016).

We also found that incidences of footpad dermatitis and digital ulcers were also significantly lower in animals fed on the HC-supplemented diet in both the challenged and non-challenged groups, suggesting that the HC-supplemented diet protects against lesions in the presence and also in the absence of coccidiosis (*Eimeria* spp.) (Nogareda et al. 2016). Footpad dermatitis characterized by inflammation and ulcers on the footpad and toes is common in poor litters and can be caused by chemicals, genetic predisposition, immunosuppressive diseases, and poor nutrition (Miljkovic et al. 2012). The animals walk on the wet litter, and the outer layers of their skin begin to soften. The litter produces friction between the soft footpad and the floor, and the outer layers erode to cause the lesions. We found that incidences of footpad dermatitis and digital ulcers were significantly lower in animals fed on the HC-supplemented diet in both the challenged and non-challenged groups, suggesting that the HC-supplemented diet protects against lesions in the presence and also in the absence of coccidiosis (Nogareda et al. 2016). Our results show that carotenoid-enriched corn can be used to maintain poultry health and immunity, thus reducing footpad dermatitis and ulcer counts on feet due to the more productive inflammatory responses to lesions and secondary infections. Our results also demonstrate that carotenoid-rich corn incorporated into commercial poultry diets can maintain animal health and confer nutritional value to poultry products without the use of expensive feed additives.

8.9 High-Carotenoid Corn in Egg Production

The biofortified corn varieties HC and BKT, which are rich in carotenoids and ketocarotenoids, respectively, were evaluated in feeding trials with laying hens to assess productivity and quality parameters in egg production and to investigate the fate and distribution of carotenoids in specific tissues, as well as in the eggs. Four diets supplemented with different types of corn (which represented a 62% of the total feed) were assessed: HC, BKT, their near-isogenic line (M37W), and a commercial yellow corn line (supplemented with 3 mg retinol per kg feed). These were evaluated in animal feeding trials with 32 laying hens (ISA Brown). Hens were fed on the M37W-supplemented diet for 12 days before starting the trial to ensure the depletion of carotenoid content in the egg yolk. The M37W-supplemented diet was then changed to the experimental diets, and the trial lasted 20 additional days (Moreno et al. 2016).

Biofortified corn diets, which were nutritionally equivalent to the other diets, except for carotenoid and ketocarotenoid levels, did not adversely affect the health of the hen. The feed conversion ratio (FCR), which is defined as the amount (g) of feed required to produce a gram of egg, was lower in HC- and BKT-supplemented diets (1.79 and 1.94, respectively) compared with M37W and commercial supplemented diets (2.22 and 2.06, respectively), resulting in a better efficiency in terms of egg production. The egg quality parameters evaluated (egg weight, breaking strength, albumen height, Haugh units, and shell thickness) showed similar results among diets (Moreno et al. 2016).

The yolk color is considered the most important quality parameter, and significant differences were found among diets. According to the DSM scale, eggs laid by hens fed on BKT, and Ph-3-supplemented diets had the highest values (11.38 and 10.08, respectively) compared with eggs laid by hens fed on M37W and commercial diets (1.25 and 4.22, respectively). Similar differences were found when yolk colors were measured by the

CIELAB trichromatic system as lightness (L*), redness (a*), and yellowness (b*). Yolks from eggs laid by hens fed on commercial and M37W-supplemented diets had the highest lightness (56.8 ± 0.70 and 57.9 ± 0.60, respectively) compared with those from hens fed on HC- and BKT-supplemented diets (51.7 ± 0.55 and 49.4 ± 0.94, respectively). The highest redness was measured in yolks from eggs laid by hens fed on biofortified corn diets (13.6 ± 0.62 and 10.8 ± 0.43 for BKT and HC, respectively) compared with those from hens fed on commercial and M37W-supplemented diets (3.6 ± 0.18 and − 1.16 ± 0.07). Regarding yellowness, yolks from eggs laid by hens fed on the commercial supplemented diet had the highest value (25.4 ± 2.38), followed by those from hens fed on HC-, BKT-, and M37W-supplemented diets (22.7 ± 1.41, 17.4 ± 0.89 and 9.7 ± 0.95, respectively) (Moreno et al., personal communication). Yolk colors are shown in Fig. 8.3.

Carotenoid analysis confirmed a correlation between carotenoid content in the experimental diets (Table 8.3) and carotenoid content in the yolks (Table 8.4). The total carotenoid content as well as provitamin A carotenoids (β-carotene and β-cryptoxanthin) were much higher in the HC-supplemented diet compared with the other diets. Therefore, eggs laid by those hens had significantly higher total carotenoid content and provitamin A carotenoids. The BKT-supplemented diet was the only one which contained astaxanthin and other ketocarotenoids (4.42 and 2.09 μg/g freeze-dried feed, respectively). Thus, only hens fed on the BKT-supplemented diet laid eggs accumulating astaxanthin and other ketocarotenoids (6.56 and 1.69 μg/g freeze-dried yolk, respectively).

Fig. 8.3 Yolks from eggs laid by hens fed on the experimental diets (commercial and BKT-, HC-, and M37W-supplemented diets from left to right) compared with DSM color scale

Carotenoid content in yolks was higher than carotenoid content in feed, suggesting that they were transferred to the eggs against a concentration gradient. This enrichment was higher when hens were fed on biofortified corn diets rather than on the commercial diet. Therefore, analyses of provitamin A (PVA) and non-provitamin A (non-PVA) carotenoids were separately performed to elucidate the mechanism of carotenoid accumulation in the yolk (Tables 8.3 and 8.4). Non-PVA carotenoids accumulated in the egg, doubling the initial concentration in the feed, particularly when biofortified diets were supplied, while PVA carotenoids were depleted by up to 50% on their way to the egg (Moreno et al. 2016).

This difference in carotenoid distribution could be explained by the conversion of PVA carotenoids into retinol and its diversion to the liver. Retinol levels in hens fed on commercial and HC-, BKT-, and M37W-supplemented diets were 1397, 1790, 1454, and 380 μg/g freeze-dried, respectively, in the liver, and 18.0, 21.08, 23.69, and 15.4 μg/g freeze-dried, respectively, in the egg. Taking into account that the commercial diet was supplemented with 3 mg retinol per kg feed, a higher retinol accumulation was found in the liver when biofortified diets were supplied compared with M37W-supplemented diet.

The specific carotenoid profiles play a more important role in yolk pigmentation than the total carotenoid content in the egg yolks. Therefore, visual color is not always correlated with total carotenoid content in the egg yolk (Karadas et al. 2006). This has been corroborated in our study. For example, the BKT-supplemented diet increased redness due to astaxanthin and other ketocarotenoids, whereas the HC-supplemented diet supplied the highest total carotenoid content. The results demonstrate that biofortified corn diets not only increase yolk pigmentation but also provide carotenoids through a direct source. They thus not only constitute a cost-effective

Table 8.3 Total carotenoid content and provitamin A (β-carotene and β-cryptoxanthin) and non-provitamin A carotenoids in the experimental diets (μg/g freeze-dried feed)

	HC	BKT	Commercial	M37W
Total carotenoid content	31.05	13.81	9.22	0.84
Provitamin A carotenoids	6.47	1.6	0.68	0.23
Non-provitamin A carotenoids	24.58	5.7	8.54	0.61

Table 8.4 Total carotenoid content, provitamin A (β-carotene and β-cryptoxanthin), and non-provitamin A carotenoids in the yolks (μg/g freeze-dried yolk)

	HC	BKT	Commercial	M37W
Total carotenoid content	57.5	26.18	11.54	1.81
Provitamin A carotenoids	3.09	0.46	0.33	0.13
Non-provitamin A carotenoids	54.41	17.47	11.21	1.68

alternative to feed supplementation in the poultry industry but also serve as a good model for carotenoid metabolism in humans.

8.10 Mice Fed on a Diet Enriched with Genetically Engineered High-Carotenoid Corn Show No Sub-acute Toxic Effects and No Sub-chronic Toxicity

The assessment of nutritionally enhanced varieties created by genetic engineering or otherwise must include (among other tests) compositional analysis, laboratory feeding trials in animals to test sub-chronic toxicity, as well as tests for allergenicity and nutritional assessments (Kuiper et al. 2001; Konig et al. 2004). As part of the development process for genetically engineered crops and following the European Food Safety Authority (EFSA) recommendations, high-carotenoid (HC) corn must be tested in whole food/feed sub-chronic animal feeding studies to ensure that there are no adverse effects, and potential allergens must be identified. A compositional analysis showed no nutritionally relevant differences between HC corn and its near-isogenic line M37W in terms of major constituents. Indeed, the only difference was the expected higher levels of novel carotenoids in HC corn (Arjo et al. 2012). These nutritional differences represent the intended effects of the genetic modification and therefore do not constitute "unintended effects" that the safety tests are ostensibly designed to identify (Arjo et al. 2012).

Diets enriched with HC corn had no adverse effects on mice, did not induce any clinical signs of toxicity, and did not contain known allergens. In order to identify short-term toxic effects, a 28-day toxicity assessment was performed in mice comparing the composition of HC corn and its near-isogenic line M37W. After 28 days, the male body weight was 23.88 ± 1.82 g in the reference diet group, 25.24 ± 0.47 g in the M37W group, and 24.81 ± 0.29 g in the HC corn group. The corresponding female body weights were 20.28 ± 1.33, 19.13 ± 1.32, and 20.69 ± 0.74 g, respectively. This experiment showed no short-term sub-acute evidence of diet-related adverse health effects in mice and no difference in clinical markers (food consumption, body weight, organ/tissue weight, hematological and biochemical blood parameters, and histopathology) compared with mice fed on a control diet (conventional varieties of the same crop) (Arjo et al. 2012).

A subsequent 90-day sub-chronic feeding study was carried out to determine any adverse effects caused by repeated exposure over a longer period. After 13 weeks, there was no statistically significant difference in either food consumption or body weight among the three diet groups when comparing the whole groups or individual sexes, although males fed on the HC corn diet were on average marginally heavier (27.76 ± 0.75 g) than

their counterparts in the other two groups (27.23 ± 1.01 g for the reference diet, 27.04 ± 1.13 g for the wild-type corn diet). The females in the reference, wild-type, and HC diet groups weighed 21.94 ± 1.44 g, 22.39 ± 1.42 g, and 22.24 ± 0.99 g, respectively. This experiment again showed no indications of toxicity in terms of biochemical markers and hematological parameters compared with mice fed on control diets. No significant differences were measured between the HC corn-fed group and control group in terms of absolute organ weight, or organ weight relative to brain or body weight, changes which often indicate hepatocellular, myocardial, adrenal gland, and renal tubular hypertrophy, neurotoxicity in the brain, and toxicity-related alterations in the reproductive or lymphoid organs. For these assays, Arjo et al. (2012) measured the weight of the adrenals, brain, epididymis, heart, kidneys, liver, ovaries, spleen, testes, thymus, and uterus. In addition, no histopathological anomalies specific to the HC corn diet were observed.

In conclusion, the genetically engineered HC corn did not show any unintended effects in animal feeding trials designed to evaluate sub-acute and sub-chronic toxicity, tests that are although not mandated by law in most jurisdictions will facilitate public acceptance of HC corn for human consumption. Diets prepared with HC corn were palatable, nutritious, and safe for animals. This should pave the way for human trials and the eventual deployment of HC corn in developing countries to help combat micronutrient deficiencies among populations that subsist on a predominantly cereal-based diet.

8.11 Engineered Maize as a Source of Astaxanthin: Processing and Application as Fish Feed

Astaxanthin, a ketocarotenoid used in salmon and trout feeding, is necessary for achieving a pink flesh coloration. Currently, most of the astaxanthin used in aquaculture is synthetic (Moretti et al. 2006). Only a small number of biological sources are available, including the bacterium *Paracoccus carotinifaciens*, the alga *Haematococcus pluvialis*, and the fungus *Xanthophyllomyces dendrorhous* (Ambati et al. 2014), which are not sufficient to meet the global astaxanthin market. An alternative source is provided by the extension of the carotenoid pathway in maize for the synthesis of astaxanthin through metabolic engineering (Breitenbach et al. 2016). A maize line expressing a hydroxylase and a ketolase gene leading to the synthesis of astaxanthin, in addition to a gene to knock down lycopene ε-cyclase and phytoene synthase 1, was crossed into a high-oil hybrid (NLS76) (Farre et al. 2016). The prevalent accumulated carotenoid in the kernels was astaxanthin, reaching ca: 60% of the total carotenoids. The resulting maize seeds containing astaxanthin were evaluated as a feed supplement source for rainbow trout.

A large-scale extraction process was established, and the isomeric composition of the astaxanthin product was determined, identifying the astaxanthin from this transgenic maize as the 3S, 30S enantiomer. The geometrical isomers were 89% all-E, 8% 13-Z, and 3% 9-Z. The astaxanthin concentration step involving multiple-phase partitioning steps was implemented to remove 90% of the oil provided an oily astaxanthin preparation for use as a fish feed ingredient. The fish were fed with astaxanthin supplement from the start of the trial with a lower dose of 7 mg/g feed, which was increased tenfold in the astaxanthin feed to 72 mg/g after 35 days to a concentration ensuring a maximum pigmentation effect. In contrast to the fillets from the astaxanthin-free feed, the application of astaxanthin resulted in a strong pink pigmentation. Pigmentation properties of the maize produced natural astaxanthin incorporated to 3.5 μg/g DW in the trout fillet resembling that of chemically synthesized astaxanthin. The only detectable carotenoid in the fillet of the control-diet fish was a small amount of zeaxanthin. By comparing the relative carotenoid composition in feed, flesh, and feces, a preferential uptake of zeaxanthin and 4-keto zeaxanthin over astaxanthin was observed (Breitenbach et al. 2016).

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References

Ambati RR, Moi PS, Ravi S, Aswathanarayana RG (2014) Astaxanthin: sources, extraction, stability, biological activities and its commercial applications – a review. Mar Drugs 12:128–152

Arjo G, Capell T, Matias-Guiu X, Zhu C, Christou P, Pinol C (2012) Mice fed on a diet enriched with genetically-engineered multivitamin corn show no sub-acute toxic effects and no sub-chronic toxicity. Plant Biotechnol J 10:129–141

Bai C, Twyman RM, Farre G, Sanahuja G, Christou P, Capell T, Zhu C (2011) A golden era-pro-vitamin A enhancement in diverse crops. Vitro Cell Develop Biol Plant 47:205–221

Berman J, Zorrilla-Lopez U, Farre G, Zhu C, Sandmann G, Twyman RM, Capell T, Christou P (2015) Nutritionally important carotenoids as consumer products. Phytochem Rev 98:85–91

Breitenbach J, Nogueira M, Farre G, Zhu C, Capell T, Christou P, Fleck G, Focken U, Fraser PD, Sandmann G (2016) Engineered maize as a source of astaxanthin: processing and application as fish feed. Transgenic Res 25:785–793

Castaneda MP, Hirschler EM, Sams AR (2005) Skin pigmentation evaluation in broilers fed natural and synthetic pigments. Poult Sci 84:143–147

D'Ambrosio DN, Clugston RD, Blaner WS (2011) Vitamin A metabolism: an update. Nutrients 3:63–103

Decourcelle M, Perez-Fons L, Baulande S, Steiger S, Couvelard L, Hem S, Zhu C, Capell T, Christou P, Fraser P, Sandmann G (2015) Combined transcript, proteome and metabolite analysis of transgenic maize seeds engineered for enhanced carotenoid synthesis reveals pleiotropic effects in core metabolism. J Exp Bot 66:3141–3150

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

Farre G, Bai C, Twyman RM, Capell T, Christou P, Zhu C (2011) Nutritious crops producing multiple carotenoids – a metabolic balancing act. Trends Plant Sci 16:532–540

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

Farre G, Blancquaert D, Capell T, Van Der Straeten D, Christou P, Zhu C (2014) Engineering complex metabolic pathways in plants. Annu Rev Plant Biol 65:187–223

Farre G, Twyman RM, Christou P, Capell T, Zhu C (2015) Knowledge-driven approaches for engineering complex metabolic pathways in plants. Curr Opin Biotechnol 32:54–60

Farre G, Perez-Fons L, Decourcelle M, Breitenbach J, Hem S, Zhu C, Capell T, Christou P, Fraser PD, Sandmann G (2016) Metabolic engineering of astaxanthin biosynthesis in maize endosperm and characterization of a prototype high oil hybrid. Transgenic Res 25:477–489

Fudge AM (2000) Laboratory medicine. Avian and exotic pets. (Ed. Fudge AM). Saunders Company, Philadelphia

Harrison GJ, Lightfoot TL (2006) Clinical avian medicine. (Eds. Harrison GJ, Lightfoot TL). Spix Publishing, Palm Beach

Karadas F, Grammenidis E, Surai PF, Acamovic T, Sparks NHC (2006) Effects of carotenoids from lucerne, marigold and tomato on egg yolk pigmentation and carotenoid composition. Br Poult Sci 47:561–566

Konig A, Cockburn A, Crevel RWR, Debruyne E, Grafstroem R, Hammerling U, Kimber I, Knudsen I, Kuiper HA, Peijnenburg AACM, Penninks AH, Poulsen M, Schauzu M, Wal JM (2004) Assessment of the safety of foods derived from genetically modified (GM) crops. Food Chem Toxicol 42:1047–1088

Kuiper HA, Kleter GA, Noteborn HP, Kok EJ (2001) Assessment of the food safety issues related to genetically modified foods. Plant J 27:503–528

Miljkovic B, Skrbic Z, Pavlovski Z, Lukic M, Ivetic V, Kureljusic B, Petricevic V (2012) Foot-pad dermatitis in commercial broilers. Biotechnol Anim Husb 28:835–843

Moreno JA, Díaz-Gómez J, Nogareda C, Angulo E, Sandmann G, Portero-Otin M, Twyman RM, Capell T, Zhu C, Christou P (2016) The distribution of carotenoids in hens fed on biofortified maize is influenced by feed composition, absorption, resource allocation and storage. Sci Rep 6:35346

Moretti VM, Mentasti T, Bellagamba F, Luzzana U, Caprino F, Turchini GM, Giani I, Valfre F (2006) Determination of astaxanthin stereoisomers and colour attributes in flesh of rainbow trout (*Oncorhynchus mykiss*) as a tool to distinguish the dietary pigmentation source. Food Addit Contam 23:105610–105663

Naqvi S, Zhu C, Farre G, Ramessar K, Bassie L, Breitenbach J, Conesa DP, Ros G, Sandmann G, Capell T, Christou P (2009) Transgenic multivitamin corn through biofortification of endosperm with three vitamins representing three distinct metabolic pathways. Proc Natl Acad Sci USA 106:7762–7767

Naqvi S, Ramessar K, Farre G, Sabalza M, Miralpeix B, Capell T, Zhu C, Christou P (2011a) High value products from transgenic maize. Biotechnol Adv 29:40–53

Naqvi S, Zhu C, Farre G, Sandmann G, Capell T, Christou P (2011b) Synergistic metabolism in hybrid corn indicates bottlenecks in the carotenoid pathway and leads to the accumulation of extraordinary levels of the nutritionally important carotenoid zeaxanthin. Plant Biotechnol J 9:384–393

Nogareda C, Moreno J, Angulo E, Sandmann G, Portero M, Capell T, Zhu C, Christou P (2016) Carotenoid-enriched transgenic corn delivers bioavailable carotenoids to poultry and protects them against coccidiosis. Plant Biotechnol J 14:160–168

Pretorius B, Schonfeldt HC (2013) Effect of different maize meal diets on growth and vitamin A: case-study on chickens. Food Chem 140:520–525

Ricroch AE, Berg JB, Kuntz M (2011) Evaluation of genetically engineered crops using transcriptomic, proteomic, and metabolomics profiling techniques. Plant Physiol 155:1752–1761

Sandmann G (2001) Genetic manipulation of carotenoid biosynthesis: strategies, problems and achievements. Trends Plant Sci 6:14–17

Zanga D, Capell T, Zhu C, Christou P, Thangaraj H (2016) Freedom-to-operate analysis of a transgenic multivitamin corn variety. Plant Biotechnol J 14:1225–1240

Zhu C, Naqvi S, Gomez-Galera S, Pelacho AM, Capell T, Christou P (2007) Transgenic strategies for the nutritional enhancement of plants. Trends Plant Sci 12:548–556

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

Zhu C, Naqvi S, Capell T, Christou P (2009) Metabolic engineering of ketocarotenoid biosynthesis in higher plants. Arch Biochem Biophys 483:182–190