

High-carotenoid maize: development of plant biotechnology prototypes for human and animal health and nutrition

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Abstract

Carolight® is a transgenic maize variety that accumulates extraordinary levels of carotenoids, including those with vitamin A activity. The development of Carolight® maize involved the technical implementation of a novel combinatorial transformation method, followed by rigorous testing for transgene expression and the accumulation of different carotenoid molecules. Carolight® was envisaged as a way to improve the nutritional health of human populations that cannot access a diverse diet, but this ultimate humanitarian application can only be achieved after extensive testing for safety, agronomic performance and nutritional sufficiency. In this article, we chart the history of Carolight® maize focusing on its development, extensive field testing for agronomic performance and resistance to pests and pathogens, and feeding trials to analyze its impact on farm animals (and their meat/dairy products) as well as animal models of human diseases. We also describe more advanced versions of Carolight® endowed with pest-resistance traits, and other carotenoid-enhanced maize varieties originating from the same series of initial transformation experiments. Finally we discuss the further steps required before Carolight® can fulfil its humanitarian objectives, including the intellectual property and regulatory constraints that lie in its path.

Introduction

Carotenoids are organic pigments produced by photosynthetic organisms to absorb light energy during photosynthesis and to protect chlorophyll from damage (Armstrong and Hearst, 1996). The antioxidant activity of carotenoids is also beneficial to animals, but only a few arthropods can synthesize carotenoids *de novo* and all other animals must obtain these molecules in their diet. Carotenoids with unsubstituted β -ionone rings have vitamin A activity, i.e. they are converted into retinal and then into either retinol (by reduction) or retinoic acid (by oxidation). The four major carotenoids with vitamin A activity are β -carotene, α -carotene, β -cryptoxanthin and γ -carotene. These are collectively known as provitamin A (PVA) carotenoids and are regarded as essential dietary components. Other non-PVA carotenoids also perform specific and arguably essential roles in the body, e.g. lutein and zeaxanthin accumulate in the retinal macula where they function to absorb blue and near-ultraviolet light. The provision of adequate quantities of carotenoids in the diet is essential, but many staple foods lack sufficient amounts of carotenoids and vitamin A deficiency is therefore prevalent in developing countries that rely on predominantly cereal-based diets with little access to fresh fruit and vegetables.

Carotenoids are synthesized by all plants and many bacteria – the biosynthesis pathway is shown in detail in **Figure 1**. The first committed step in carotenoid biosynthesis is the conversion of geranylgeranyl diphosphate (GGPP) into phytoene by phytoene synthase (PSY), and this is recognized as a major pathway bottleneck. Indeed, this is precisely the reason for the near complete lack of carotenoids in rice grains and in the South African elite white-endosperm maize inbred M37W, which has a central role in the development of high-carotenoid maize as discussed in detail below. Phytoene is desaturated and isomerized in several steps to form lycopene, but one bacterial enzyme (CRTI) can accomplish all these reactions. Lycopene is then cyclized at each end by lycopene β -cyclase (LYCB, bacterial equivalent CRTY) to form β -carotene, or at one end by lycopene ϵ -cyclase (LYCE) and at the other by LYCB to form α -carotene. Both α -carotene and β -carotene can be converted into downstream products by carotene hydroxylases, such as β -carotene hydroxylase (BCH) and carotenoid ϵ -hydroxylase. In the β -carotene pathway this yields β -cryptoxanthin, which is further converted by the same enzyme into zeaxanthin, whereas in the α -carotene branch the conversion yields lutein, the natural pathway endpoint. Zeaxanthin enters the xanthophyll cycle through the activity of zeaxanthin epoxidase yielding violaxanthin. A de-epoxidase can catalyze the back reaction (Farré et al., 2010b). Although higher plants synthesize different hydroxylated carotenoids they in general are not able to produce ketocarotenoids such as astaxanthin. An exception is *Adonis* species which accumulate astaxanthin in their petals.

The absence of carotenoids in rice grains has been addressed by the expression of two carotenogenic transgenes in the endosperm to generate Golden Rice, which accumulates β -carotene: *Zmpsy1* (maize phytoene synthase) and *crtI* (phytoene desaturase) from the bacterium *Pantoea ananatis* (formerly called *Erwinia uredovora*) (Paine et al., 2005). In maize, we used M37W as a baseline because it only produces trace amounts of carotenoids in the endosperm. This variety is culturally preferred in sub-Saharan Africa over yellow maize varieties that produce moderate levels of non-PVA carotenoids and also provides a blank canvas to develop carotenoid-enriched varieties because the accumulation of different carotenoids is immediately apparent through the production of colored endosperm.

Generation of high carotenoid maize varieties with different carotenoid profiles

The path towards high-carotenoid biofortified maize began when we transformed M37W with multiple carotenogenic transgenes using standard novel combinatorial transformation procedure, in which immature maize embryos are bombarded with DNA-coated metal particles (Ramessar et al., 2008). All plants were also transformed with the selectable marker *bar*, which encodes phosphinothricin acetyltransferase and confers herbicide tolerance for selection. The resulting library of transformants (Zhu et al., 2008) included a range of genotypes representing different combinations of up to five metabolic input transgenes, all under the control of endosperm-specific promoters: *Zmpsy1*, *PacrtI* (*P. ananatis* phytoene desaturase), *Glycb* (*Gentiana lutea* lycopene β -cyclase), *Glbch* (*G. lutea* β -carotene hydroxylase) and *ParactW* (*Paracoccus* sp. β -carotene ketolase). The plants showed a diverse spectrum of endosperm color phenotypes representing the effects of different combinations of transgenes superimposed on the endogenous carotenoid biosynthesis pathway (**Figure 2**). One of the resulting transformants carried *Zmpsy1* and *PacrtI*, the first two genes in the carotenoid pathway, and the endosperm produced extraordinary levels of β -carotene (up to 60 $\mu\text{g/g}$ DW). This became the first-generation Carolight[®] variety. Another was found to carry all five of the metabolic input transgenes in addition to the selectable marker. The inclusion of *ParactW* among the five transgenes meant that this was the first transgenic maize variety to produce ketocarotenoids (36 $\mu\text{g/g}$ DW, including 4.46 $\mu\text{g/g}$ DW astaxanthin). Other events were found to contain different combinations of two, three or four of the input transgenes in addition to the selectable marker. Second-generation experiments were carried out to increase the levels of astaxanthin by knocking down endogenous lycopene ϵ -cyclase expression in the M37W endosperm while introducing *Zmpsy1*, *sBrcrTZ* (synthetic *Brevundimonas* sp. SD212 β -carotene hydroxylase), and *sCrbkt* (synthetic truncated β -carotene ketolase from the alga *Chlamydomonas reinhardtii*) codon optimized for plants (Farré et al., 2016). The best-performing line in terms of astaxanthin production

(line BKT) was crossed with the high-oil NSL76 line to generate the hybrid NSL76-BKT which accumulated 45% of total seed carotenoids as astaxanthin, up to 17 $\mu\text{g/g}$ DW (Farré et al., 2016). The combination of astaxanthin, starch and high oil content makes NSL76-BKT particularly suitable as a feed component, as discussed in more detail below.

Agronomic performance in the greenhouse and field

Novel crops created with enhanced traits should be tested against their near-isogenic wild-type counterparts under a variety of environmental conditions to ensure that performance (in this case the accumulation of carotenoids) is maintained in different environments that are likely to be encountered following commercial release. This is particularly important with transgenic crops because early development is restricted to greenhouse cultivation and rigorous field testing is therefore necessary to determine the impact of variable temperature, light intensity, humidity and weather in the field (collectively abiotic stress) as well as exposure to pests and diseases (collectively biotic stress). We therefore evaluated Carolight[®] plants under greenhouse and field conditions and compared the carotenoid content and accumulation in developing endosperm over time. We also measured endogenous carotenogenic gene expression during endosperm development. Minor differences in the endosperm carotenoid composition were measured during early development, but there were no significant differences when we compared the mature endosperm of plants from the greenhouse and field, in terms of either the total carotenoid content or the carotenoid profile (Zanga et al., in press).

Conventional breeding and genetic engineering can affect performance traits such as yield and biomass accumulation in a positive (Subedi and Ma, 2007) or negative (Elmore et al., 2001a; 2001b) manner, or the effect may be neutral (Marra et al., 2004; Laserna et al., 2012). Carotenoid enrichment is a form of metabolic engineering, so it is possible that the precursors and energy directed towards the synthesis of carotenoids may result in a trade-off with other traits due to competition for resources. We therefore set up a field trial to compare performance of Carolight[®] and M37W over three years. We applied two fertilization regimes to both varieties (no fertilization and 150 kg nitrogen ha^{-1}). There were no significant differences in yield between Carolight[®] and its isogenic line under either of the nitrogen treatments, nor was there any evidence of a significant nitrogen \times genotype interaction (Zanga et al. in press). Furthermore, there were no significant genotypic differences in the leaf oxygen isotopic composition ($\delta^{18}\text{O}$) at any developmental stage, suggesting the two lines performed similarly in terms of stomatal control of water loss, which is an indicator of drought resistance (Cabrera-Bosquet et al., 2009). There was a significant difference in

$\delta^{18}\text{O}$ between developmental stages in both genotypes regardless of the nitrogen treatment, which indicated greater stomatal control during flowering when crop water demand is highest.

Resistance to pests and pathogens

The major intended application of Carolight® is the provision of a nutritionally improved staple crop for human consumption, so it was important to introduce further traits into this variety to make it compatible with current maize cultivation practices and save pesticide application and consequent residues as much as possible. We therefore introduced the *Bacillus thuringiensis* (Bt) *cry1Ac* gene, which is widely used in commercial maize varieties to provide resistance against lepidopteran pests, particularly corn borers. Transgenic maize lines transformed with *cry1Ac* were produced from the M37W genetic background and the resulting plants were characterized to confirm transgene expression at the mRNA and protein levels, to ensure that the Bt toxin was produced at sufficient amounts for effective insect control. This resulted in the identification of an elite event which was crossed with homozygous Carolight® plants. The hybrid progeny were screened for the presence of both *cry1Ac* and the carotenogenic genes, and positive lines were self-pollinated to generate homozygous lines for field testing. Tests confirmed that the enhanced Carolight® plants expressing *cry1Ac* were protected from common lepidopteran maize pests such the European corn borer (*Ostrinia nubilalis*) and the Mediterranean corn borer (*Sesamia nonagrioides*) with similar rates of mortality compared to the commercial event MON810, which contains the related Bt gene *cry1Ab* (Zanga et al., in preparation).

These trials were important because little is known about the effect of biofortified crops such as Carolight® on herbivore development and behavior. For example, surplus vitamin C in the diet of herbivorous insects can have both positive and negative effects, the latter including delayed growth, lower weight, lower fecundity and a shorter lifespan (Goggin et al., 2010). However, it is unknown whether the levels of vitamin A and other carotenoids affect insect pests and whether the current range of insecticidal transgenes would work in a crop that has been engineered to increase carotenoid levels in the endosperm. Carotenoids are used by insects as visual pigments in their body markings, as antioxidants, and for the synthesis of certain hormones, but the relationship between carotenoids in food and their functions in insects have not been investigated (Heath et al., 2013). The oxidative cleavage of carotenoids into volatile apocarotenoids is likely to affect insect behavior because these act as semiochemicals, some of which are attractive others repulsive (Caceres et al., 2016). Such repellent effects may explain why the maize stem borer (*S. nonagrioides*) found

Carolight® plants less attractive as a place to lay eggs than isogenic control plants (Cruz and Eizaguirre, 2015).

Several experiments were therefore conducted to determine whether the higher levels of carotenoids in Carolight® affected the development of two maize pests: the abovementioned European corn borer, which penetrates the stalks and cobs but also feeds on leaves, and the armyworm moth (*Mythimna unipuncta*), which feeds mainly on the leaves. Larvae from both species were allowed to feed on Carolight® leaves or leaves from the near-isogenic M37W control for 14 days, and three parameters were investigated: larval mortality, leaf consumption, and larval weight. The results were similar for both pest species. There were no significant differences in mortality after 14 days but both species consumed less leaf material when presented with the Carolight® line although this did not significantly affect larval weight. This was interesting because the direct measurement of carotenoid levels in the leaves revealed no differences in carotenoid content (the transgenes are expressed under the control of endosperm-specific promoters and carotenoid biosynthesis is only modulated in the endosperm). However, the Carolight® leaves were less pigmented than the M37W leaves suggesting a knock-on effect of carotenoid metabolism in the seeds which affects the leaves and, more specifically, how attractive they are to insect pests. This difference in pigmentation was not sufficient to cause a change in photosynthetic performance as reflected in grain yield (discussed in the previous section). The conclusion from these trials is therefore that the presence of additional carotenoids in the Carolight® variety per se does not change the mortality of major pest insects, but does result in the consumption of less leaf biomass at the larval stage.

As well as the direct negative effects on plant health, the damage caused by insect pests also encourages the colonization of maize grains by filamentous fungi. Some of these opportunistic fungi produce mycotoxins, secondary metabolites that can cause illness in both humans and livestock. Maize seeds are often contaminated with fumonisins produced by *Fusarium verticillioides* and *F. proliferatum*, although other mycotoxins such as aflatoxins may be found alone or together with fumonisins in maize (Marín et al., 2013). Several studies have reported a significant reduction in pest damage, disease symptoms and fumonisin levels in maize hybrids that express Bt genes (Díaz-Gómez et al., 2015). However, the effect of carotenoids on mycotoxin contamination has not yet been studied in detail.

The field tests were carried out for three consecutive seasons using M37W and Carolight® without the additional Bt trait (to allow us to test the effect of the carotenoids alone, in the absence of the known beneficial effects of Bt). The grain of both varieties was tested for the prevalence of fungal contamination and the accumulation of different mycotoxins. There were no significant differences

in the extent of fungal infection (expressed as the proportion of infected grains), and as expected the dominant genus in all three seasons was *Fusarium* and the most common species was *F. verticillioides*. *Aspergillus* species were not identified in any of the tested samples, and accordingly there was no sign of contamination with aflatoxins. Due to the presence of *Fusarium* spp. there was fumonisin contamination in both Carolight® and M37W, but the proportion of infected grains was substantially higher in the M37W variety (1.4-fold, 2.4-fold and 2-fold more in the 2013, 2014 and 2015 seasons, respectively). It therefore seems likely that the more abundant carotenoids in the Carolight® grains either reduce the amount of damage caused by insects and thus limit the opportunity for fungal contamination (which would concur with the feeding assays discussed above), or the antioxidant activity of the carotenoids has a direct inhibitory effect on fungal growth, fumonisin biosynthesis or accumulation in maize plants. Indeed, both mechanisms may operate simultaneously (Díaz-Gómez et al., 2016).

Animal feeding trials as a first step towards a human nutritional product

As discussed above, Carolight® maize is envisaged as a technology-based solution to improve the range of micronutrients available to malnourished human populations. Therefore, in line with other novel crops, transgenic or otherwise, intended for commercial release Carolight® must be tested according to internationally established guidelines to ensure safety. The assessment of novel crops must include compositional analysis, laboratory animal feeding trials to test sub-chronic toxicity/allergenicity, and nutritional assessment (Kuiper et al., 2001; König et al., 2004; EFSA 2011a; 2011b).

Carolight® was initially tested in the laboratory to confirm its substantial equivalence to the near-isogenic line M37W. Compositional analysis showed no nutritionally-relevant differences between Carolight® and M37W in terms of its major constituents, other than the intended modification of the carotenoid content (Arjó et al., 2012). This was followed by a 90-day sub-chronic toxicity study in mice, which was designed to address the consequences of repetitive exposure for a duration considered long enough to reveal the effects without confounding age-related effects (EFSA 2011a). During this feeding trial, mice reared on two nutritionally balanced diets identical in all respects apart from the presence of either Carolight® or M37W showed no differences in their rates of food consumption or body weight gain, which are generally recognized as broad indicators of health and wellbeing. This provided strong evidence that the consumption of Carolight® does not induce unintended nutritional effects. Similarly, there were no significant differences between the diet groups in terms of clinical observations, hematological parameters, biochemical markers and

absolute or relative changes in organ weight. In summary, Carolight® was found to be compositionally substantially equivalent to M37W and to satisfy current requirements to demonstrate the absence of sub-chronic toxicity (Arjó et al., 2012). No single animal model addresses all potential sources of toxicity (Gephart et al., 2001) but a positive outcome following a 90-day sub-chronic toxicity test provides strong supporting evidence for safety (Ricroch, 2013).

The beneficial effects of a carotenoid-rich diet are well known (Pérez-Massot et al., 2013) so the next step in the development of Carolight® was to investigate its effects as the primary dietary component in preclinical models of chronic diseases, beginning with its impact on insulin sensitivity in a mouse model of insulin resistance and obesity induced by a high-fat diet. Overweight and obese individuals carry a higher risk of type-2 diabetes (Pan et al., 2013; Breen et al., 2014). Studies in both experimental animal models and humans have shown that dietary fat composition influences insulin sensitivity, i.e. saturated fats promote high plasma insulin levels (a surrogate for lower insulin sensitivity) whereas unsaturated fats help to improve insulin sensitivity (Riccardi et al., 2004). Interestingly, several studies have shown that obese individuals often face malnutrition, in terms of vitamin A deficiency (Calder et al., 2011; Wolf, 2010; Gunanti et al., 2014). Insulin resistance can be reversed by lifestyle changes like the adoption of a healthy diet before the onset of overt diabetic symptoms, including the acquisition of sufficient vitamin A (Macotela et al., 2011). Obesity also increases the risk of several other chronic diseases, including hypercholesterolemia, hypertriglyceridemia, cardiovascular disease, non-alcoholic fatty liver disease (NAFLD), hypertension/stroke, bone disorders and certain forms of cancer (Greenberg and Obin, 2006). Preclinical tests indicated that the inclusion of Carolight® rather than M37W in the diet improved systemic glucose homeostasis and the LDL/HDL cholesterol ratio in obese mice by increasing the sensitivity of skeletal muscle and adipose tissue to glucose uptake. The mixture of carotenoids contained in the Carolight® endosperm matrix therefore appears to achieve health-promoting effects in obese mice, and the pathophysiological data from this study could facilitate the rational development of nutrition-based treatments for insulin resistance. Further experiments are required to determine the precise effects of Carolight® maize in this mouse model and the underlying mechanism (Arjó, 2014).

Insulin resistance is usually associated with NAFLD, which includes hepatic macrovesicular steatosis with inflammation that can ultimately lead to cirrhosis and eventually hepatocellular carcinoma (Armstrong et al., 2014). The molecular pathophysiology of NAFLD remains unclear, but the most widely accepted theory involves an initial increase in triglyceride accumulation followed by enhanced cellular responses such as oxidative stress and the production of pro-inflammatory cytokines. Nutritional approaches have been considered for the prevention and treatment of NAFLD

and retinoic acid can prevent NAFLD (Amengual et al., 2010; Liu et al., 2015; Stefanska et al., 2012). Because pro-vitamin A carotenoids in the diet are converted into retinal by carotene dioxygenase, and from there either into retinol or retinoic acid, a further preclinical feeding trial was designed in which Carolight® maize was fed to mice haploinsufficient for the phosphatase and tensin homolog gene (*PTEN*), which causes them to develop NAFLD spontaneously. When these *PTEN*^{+/-} mice were fed on Carolight® maize, lipid metabolism in the liver was modulated to reduce lipogenesis, resulting in a less severe NAFLD phenotype (Eritja et al., 2016). Although caution should be exercised when extrapolating these findings to humans, the study suggests that Carolight® maize could be developed as part of a combinational dietary intervention to reduce steatosis in patients genetically or phenotypically predisposed to NAFLD.

Applications of Carolight® maize in agriculture

Poultry production

The typical commercial poultry diet based on maize and soybean does not supply sufficient carotenoids to produce the golden skin preferred by many consumers, and does not confer additional health benefits upon the animals. Vitamin A (in the form of retinol) and natural or synthetic pigments are routinely added to poultry feed, increasing the production costs (Castañeda et al., 2005). Carolight® maize was therefore evaluated as a replacement for carotenoid additives in a laboratory feeding trial using commercial broilers (Ross 308 males) fed on diets supplemented with 58% Carolight® maize or M37W maize as a control (Nogareda et al., 2016). There were no differences between the diet groups in terms of growth, final body weight or the final weight of most organs. The exception was the bursa of Fabricius, a lymphoid gland located on the posterodorsal wall of the cloaca that regresses with sexual maturity but plays an important role in disease resistance. This was heavier in the birds fed on the diet containing Carolight® maize, but there were no histopathological abnormalities that would indicate a health problem. 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. This revealed significant differences in the color of the meat, skin and cutaneous structures such as the comb and the base of the feathers (**Figure 3**). Chickens raised on Carolight® maize were healthy and accumulated higher levels of bioavailable carotenoids in peripheral tissues, muscle, skin and fat, and also more retinol in the liver, than birds fed on control maize diets. A second similar trial was conducted under farm environment conditions. The results were identical to those we obtained in the initial laboratory trial (Nogareda et al., 2016).

Having confirmed that the Carolight® maize diet was safe in broilers and promoted carotenoid accumulation in many different organs and structures, a further trial was carried out incorporating a challenge with *Eimeria tenella*, one of several protozoan parasites that cause coccidiosis, an important disease in commercial broiler farms. Two groups of animals were fed on each of the two diets used in the first and second trials described above, resulting in four experimental groups in total. One group on each diet was challenged orally with an *E. tenella* inoculum of 24.3×10^4 sporulated oocysts (Houghton strain). During the trial, the birds were visually inspected for foot pad dermatitis and digital ulcers. After the trial, the post-challenge fecal oocyst counts were evaluated, and the birds were dissected for gross necropsy and the analysis of intestinal lesions. The birds on the Carolight® diet challenged with the parasite suffered milder symptoms than those on the M37W diet and the fecal oocyst counts were significantly lower. The incidence of foot pad dermatitis and digital ulceration was significantly lower in animals fed on Carolight® diet in both the challenged and non-challenged groups, suggesting that the high-carotenoid diet protects against lesions in both the presence and absence of coccidiosis. The incorporation of Carolight® maize into broiler diets therefore not only improves the carotenoid levels in skin and meat, but also provides a cost-effective strategy for the protection of broilers against coccidiosis without the need for carotenoid additives (Nogareda et al., 2016).

Egg production

Synthetic carotenoids or carotenoid additives such as marigold and red pepper are not only added to broiler diets but also to the feed provided to laying hens in order to achieve the characteristic yellow-orange color of egg yolks desired by consumers. As discussed above for broilers, the use of biofortified maize varieties producing additional carotenoids would provide a more cost-effective solution because no additives would be required. However, it is unclear how carotenoids are mobilized from the feed to the eggs and whether certain carotenoids are more likely to be deposited in the egg yolk or diverted to the tissues in the hen. For this reason, feeding trials were carried out using not only the Carolight® variety which is rich in carotenoids, but also the abovementioned BKT variety which is rich in ketocarotenoids and produces a darker red-orange endosperm than Carolight® maize. Hens fed on diets supplemented with either Carolight® or BKT were compared to those fed on the M37W variety and a commercial diet of yellow maize with added retinol, typical in the poultry industry. The hens were assessed for their productivity and egg quality parameters during the trial, and were then dissected for gross necropsy and to investigate the fate and distribution of carotenoids in specific tissues (including the eggs). Hens in all four diet groups were initially fed on the M37W diet for 12 days to deplete the carotenoid content of their tissues and

eggs, and then switched to the four experimental diets for the remaining 20 days. The two biofortified maize diets had the lowest feed conversion ratio (weight of feed consumed/weight of eggs produced) which means that less feed was consumed to produce the same weight of eggs, i.e. the biofortified diets were nutritionally more efficient. There were no differences among the diet groups in egg quality parameters such as average egg weight, breaking strength, albumen height, Haugh units or shell thickness, but substantial differences in terms of yolk color based on the DSM scale. Before the trial, the hens produced eggs with an average yolk DSM value of just below 10. This fell to 1.25 after 12 days on the M37W diet as the carotenoids stored by the hens became depleted. After 20 days on the four different experimental diets, eggs laid by hens fed on diets supplemented with BKT and Carolight® maize had the highest DSM values (11.38 and 10.08, respectively) compared to eggs laid by hens fed on the commercial and M37W-based diets (4.22 and 1.25, respectively) as shown in **Figure 4** (Moreno et al., submitted).

One of the key results from this feeding trial was data concerning the distribution of different carotenoids in the feed, the hens and the eggs. The four diets (Carolight®, BKT, commercial maize and M37W) had total carotenoid levels of 31.05, 13.81, 9.22 and 0.84 µg/g fd (freeze-dried tissue), respectively, whereas the total carotenoid content of the eggs from the same diet groups was 57.5, 26.18, 11.54 and 1.81 µg/g fd, respectively. These results suggested that specific carotenoids are favorably transferred to the eggs against a concentration gradient as they accumulate at higher levels in the egg compared to the initial concentration in the feed. The enhancement was most noticeable in the maize diets without retinol, including M37W which only contains low levels of carotenoids (Moreno et al., 2016). Interestingly, a more detailed analysis of the distribution of different carotenoids revealed that PVA carotenoids were diverted on their way to the egg whereas non-PVA carotenoids accumulated in the egg against the concentration gradient, doubling (lutein) or even tripling (zeaxanthin) the initial concentration in the feed. The hens appear to sequester PVA carotenoids from the maize, as well as the retinol provided in the commercial diet, whereas non-PVA carotenoids such as lutein and zeaxanthin are specifically directed to the yolk even if the hen tissues are relatively depleted. The PVA carotenoids appear to be converted into retinol and stored in the liver, because the liver retinol levels in the four diets (Carolight®, BKT, commercial maize and M37W) were 1397, 1790, 1454 and 380 µg/g fd, respectively, even though the commercial diet was directly supplemented with retinol. The provision of PVA carotenoids in biofortified maize therefore appears to be a more efficient way of providing hens with retinol than giving them retinol supplements, which may reflect the indirect manner in which PVA carotenoids are converted to retinol via retinal. Even so, the eggs produced by hens on the Carolight® diet contained more of the PVA carotenoids β-carotene and β-cryptoxanthin than the other diet groups. Uniquely, the eggs in the BKT diet group

accumulated ketocarotenoids, mainly astaxanthin, the deep red color of which explains the higher DSM score of the eggs in the BKT diet group despite the higher total carotenoid levels in the Carolight® diet group (Moreno et al. submitted).

Swine production

The National Research Council daily recommended intake of vitamin A for swine varies from 1750 IU in starters (from weaning to 25 kg) to 4000 IU in finishers (from 80 kg to market weight) (NRC, 2012). Swine convert β -carotene and other PVA carotenoids into vitamin A with only about ~30% of the efficiency seen in rats or poultry (McDowell, 2000). Dietary supplements are therefore necessary to prevent vitamin A deficiency, and feed manufacturers are authorized to recommend retinol supplements at 5 times the NRC levels (Feed additives regulation EU 2015/724). Maize is a major feed grain and a standard component of swine diets as a source of digestible energy. Carotenoid-biofortified maize varieties such as Carolight® could therefore be useful in swine nutrition by providing both a source of calories and carotenoids.

In a pilot experiment at the CEP Consortium Swine Research Centre facilities (Lleida, Spain) finishing pigs were reared on diets incorporating either 20% Carolight® maize or an equivalent amount of M37W. Two groups of 16 pigs were fed *ad libitum* on the two diets from 160 to 190 days of age. There were no significant differences in any of the production traits that were monitored during the trial (live weight, feed intake, average weight gain or feed conversion rate) agreeing with the 90-day exposure experiment in mice (Arjó et al., 2012). At 190 days, the pigs were slaughtered and samples of liver, subcutaneous fat and muscle (*longissimus dorsi* and *gluteus medius*) were collected to determine the fat content and composition. The livers from the Carolight® diet group contained three times as much all-*trans* retinoic acid as livers from the M37W group, and both the livers and the muscles from the Carolight® group contained 15-55% less fat than the control group. The subcutaneous fat was enriched for mono-unsaturated fatty acids (48.0% in the Carolight® group, 46.6% in the M37W group) particularly oleic acid (42.1% in the Carolight® group, 40.5% in the M37W group) mainly at the expense of polyunsaturated fatty acids. In contrast, the liver fat in the Carolight® group was depleted for monounsaturated fatty acids (18.4% in the Carolight® group, 22.4% in the M37W group) but accumulated more polyunsaturated fatty acids (46.8% in the Carolight® group, 42.9% in the M37W group). The lower fat content in Carolight®-fed pigs is consistent with the general finding that retinoic acid can prevent NAFLD in mice (Amengual et al., 2010; Eritja et al., 2016), which is supporting evidence for a favorable effect also in humans.

In terms of swine production, these results show that the inclusion of carotenoid-rich biofortified maize reduces the need for vitamin A supplements. The loss of intramuscular fat and lower polyunsaturated fatty acids in the subcutaneous fat has two important implications for meat quality: first, the depletion of intramuscular fat may affect the eating quality of pork because there is a positive relationship between intramuscular fat and sensory attributes; second, the depletion of polyunsaturated fatty acids reduces the risk of rancidity, particularly in processed products such as dry-cured hams and salami.

Aquaculture

Astaxanthin is the most expensive feed ingredient used in salmon and trout farming but its inclusion is necessary to achieve the aesthetic pink flesh color preferred by consumers. Most of the astaxanthin used in salmon and trout farming is synthetic (Moretti et al., 2006; Berman et al., 2015). There are only a few biological sources, such as the bacterium *Paracoccus carotinifaciens*, the alga *Haematococcus pluvialis* and the fungus *Xanthophyllomyces dendrorhous*, and their availability is limited (Ambati et al., 2014). Therefore, biological production is not sufficient to supply the global astaxanthin market, which has a current annual demand of 300 tons (Research and Markets, 2015; Berman et al., 2015). Astaxanthin produced in maize line NSL76-BKT has therefore been evaluated as replacement for synthetic astaxanthin in salmon and trout feed, in a feeding trial lasting 7 weeks (Breitenbach et al., 2016). The extracted raw material was mostly non-esterified astaxanthin, with 12% adonixanthin and 2% zeaxanthin as additional carotenoids. Isomeric analysis confirmed the exclusive presence of the 3S, 3'S astaxanthin enantiomer. The incorporation of the oily astaxanthin preparation into trout feed delivered 7 mg/kg astaxanthin in the final feed formulation for the first half of the trial, and 72 mg/kg in the last half of the trial. The pigmentation of the trout fillets was quantified by assessing hue values using a DSM color meter (**Figure 5**) and the level of astaxanthin determined by HPLC was 3.5 µg/g FDW. The comparison of relative carotenoid levels in the feed, fillets and feces revealed that zeaxanthin and adonixanthin were absorbed more efficiently than astaxanthin (Breitenbach et al., 2016).

Intellectual property and freedom to operate

Before Carolight® seed can be introduced into agricultural systems, any issues relating to intellectual property in developed and developing countries must be addressed. Patents that might impact Carolight® research, development and release in target countries must be identified in order to avoid any infringement of existing intellectual property rights. Licenses may need to be negotiated

to allow the introduction of Carolight® into agricultural systems. A freedom to operate (FTO) analysis was therefore carried out to identify granted patents and patent applications that might affect its commercialization and distribution. Although the FTO analysis was carried out by scientists rather than legal experts, thus limiting its legal value, the preliminary FTO conclusions nevertheless provide an inexpensive initial basis for a more formal FTO Opinion, which must be prepared by legally trained professionals. By deconstructing the Carolight® product into its individual components, including materials such as genetic elements, and processes such as genetic transformation (Kowalski and Kryder, 2002; Kryder et al., 2000), 26 patents or patent families that potentially affect FTO were identified (Zanga et al., 2016). These patents were identified based only on content, without screening to determine whether the patents had expired or whether the protection had been sought or granted in Europe/Spain (place of development) or South Africa/India (principal places of intended deployment). Further analysis, considering the expiry dates and geographical validity, reduced the number of relevant patent family members requiring more detailed assessment to three: PCT application WO2001088169 assigned to Monsanto Technology LLC (carotenogenic genes), and EP2297189 assigned to Athenix Corp and EP2087120 assigned to Pioneer Hi-Bred (both referring to Bt insecticidal genes). These patents should be analyzed by legal experts to determine whether the production and/or commercialization of Carolight® could infringe any intellectual property rights related to these patents. If such infringements are identified, appropriate licenses will need to be negotiated with the owners of protected materials and processes.

Regulatory constraints

Carolight® maize is a genetically-modified (GM) crop and the principal regulatory constraints therefore reflect this status. Despite the continuous growth in GM agriculture since the first commercial releases in 1995 and the widespread acknowledgements of its benefits (James, 2015), the regulatory framework for the development of new GM varieties has become increasingly difficult to negotiate, mainly reflecting the pernicious effect of public and media hostility on the political handling of GM technology (Ramessar et al., 2009). There is also a stark difference between the regulation of GM crops in North America and the European Union, with most countries in the world adopting a regulatory framework that is aligned with either one or the other. The North American system is product based, and risk assessment is based on the concept of substantial equivalence. This means that the weight of evidence must show that a new product is dangerous before commercial development can be stopped. In contrast, the EU system is process based, and risk assessment is based on the precautionary principle. This means that identical crops developed by conventional breeding and biotechnology would be regulated differently due to the different

processes involved, and the weight of evidence is required to show that a new product is safe before commercial development can be approved. The paradoxes inherent in the EU system have been widely discussed (Masip et al., 2012) and the absence of science-based reasoning has resulted in a system of completely disharmonious and incompatible regulations at the global level and even between different Member States within the EU (Ramessar et al., 2010). When the complexity of trade agreements is layered on top of this, absurd situations arise such as countries banning the cultivation of GM crops but being legally obliged to import the same products grown abroad. In practical terms, this means that GM crops developed for humanitarian reasons are not only held up by IP constraints but also by the inconsistent regulatory framework, particularly given that many developing countries follow the EU system (Farré et al., 2010a; 2011). The cost of development must therefore include a substantial budget for regulatory affairs that dwarfs the costs of developing the actual product, making it very difficult for the technology to fulfil its promise (Berman et al., 2013; Yuan et al., 2011).

Conclusions

Carolight® maize has been developed as an efficient and economical vehicle to improve the nutritional health of human populations that cannot access a diverse diet. As well as providing essential nutrients in the form of PVA carotenoids, a carotenoid-enriched diet also provides numerous health benefits due to the general antioxidant activity of these pigments, which therefore have a positive effect on the outcome of several chronic diseases and infections exacerbated by oxidative stress. The use of animal diets based on carotenoid-enriched maize not only reduces costs by removing the need for artificial additives, but also protects the animals from stress and disease, and passes the benefits of carotenoid-rich meat and dairy products to humans. The carotenoids embedded in the matrix of the maize endosperm are also assimilated more efficiently than the same nutrients supplied as extracts or retinol supplements. These benefits do not attract a penalty in terms of agronomic performance and indeed Carolight® even appears to be more resistant to insect pests and mycotoxigenic fungi than its near-isogenic counterpart. The benefits of Carolight® and other carotenoid-enriched maize varieties such as BKT nevertheless will be challenging to realize without careful negotiation of the IP landscape and also the funding necessary to address an increasingly torturous regulatory system.

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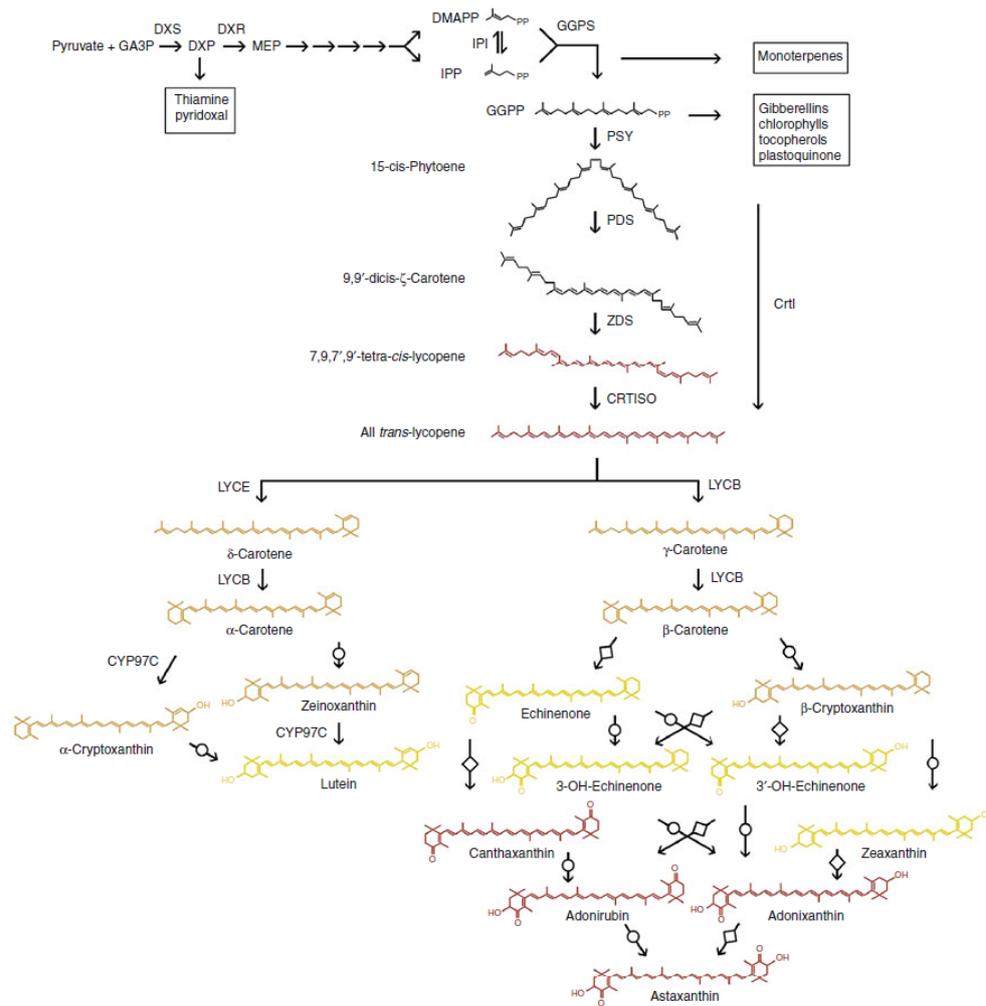


Figure 1. The extended carotenoid biosynthesis pathway in plants (Farré et al., 2010). The precursor for the first committed step in the pathway is GGPP (geranylgeranyl pyrophosphate), which is converted into phytoene by phytoene synthase (PSY, CrtB). GGPP is formed by the condensation of IPP (isopentenyl pyrophosphate) and DMAPP (dimethylallyl pyrophosphate), which are derived predominantly from the plastidial MEP (methylerythritol 4-phosphate) pathway as shown in the upper part of the figure. The pathway is linear until lycopene, involving three steps catalyzed by separate enzymes in plants but by the single, multifunctional enzyme CrtI in bacteria. Lycopene is the branch point for the α - and β -carotene pathways, which usually end at lutein and zeaxanthin, respectively, through the expression of β -carotene hydroxylases (arrows with circles). An elaborated ketocarotenoid pathway can be introduced by expressing β -carotene ketolases (arrows with diamonds) because these compete for substrates with β -carotene hydroxylases and generate diverse products. Other abbreviations: GA3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; DXR, DXP reductoisomerase; IPI, IPP isomerase; GGPS, GGPP synthase; PDS, phytoene desaturase; ZDS, ζ -carotene desaturase; CRTISO, carotenoid isomerase; LYCB, lycopene β -cyclase; LYCE, lycopene ϵ -cyclase; HydE, carotene ϵ -hydroxylase.

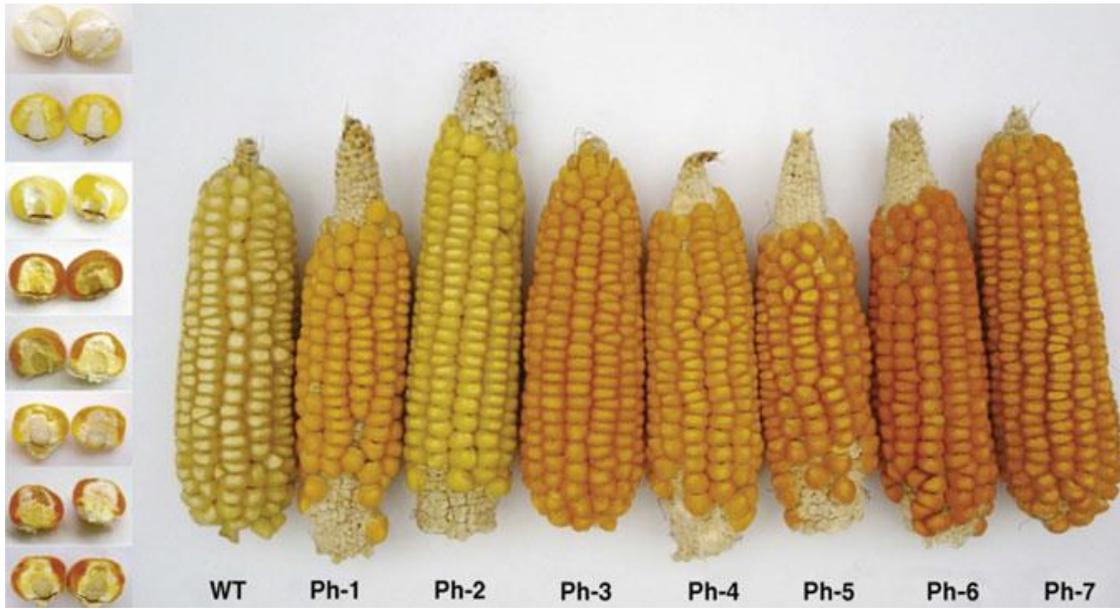


Figure 2. Seven different phenotypes based on endosperm color were obtained by combinatorial nuclear transformation (Zhu et al., 2008). The five carotenogenic genes *Zmpsy1*, *Pacrt1*, *Glycb*, *Glbch* and *ParactW* were introduced into M37W resulting in five genotypes, including Ph-3 (*Zmpsy1* + *Pacrt1*) which became Carolight®.



Figure 3. Morphological comparison of chickens fed on the M37W control diet (left images in all panels) and the Carolight® diet (right images in all panels) showing carotenoid accumulation in the skin and cutaneous structures such as the beak, crest, eyelids, and at the base of feathers (Nogareda et al., 2016).



Figure 4. Yolks from eggs laid by hens fed on four experimental diets (from left to right: commercial diet and diets supplemented with BKT, Carolight® and M37W maize, respectively) compared to the DSM color scale (Moreno et al., 2016).

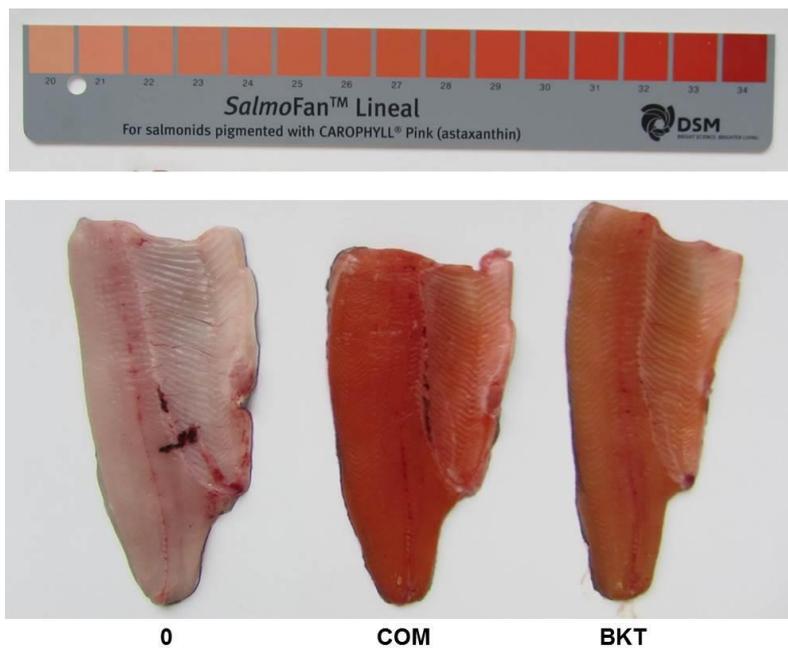


Figure 5. Trout fillets compared using the DSM scale for astaxanthin. From left to right: 0 = no astaxanthin in the feed; COM = commercial feed containing synthetic astaxanthin (CAROPHYLL); BKT = feed supplemented with BKT maize (Breitenbach et al., 2016).