

Anthocyanins in corn: a wealth of genes for human health

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Abstract Different epidemiological and preclinical studies have demonstrated that regular consumption of anthocyanin-rich foods is associated to a reduced risk of chronic diseases, such as cardiovascular diseases, cancer and obesity. However, assigning a health property to anthocyanins or other classes of flavonoids may be limited by the influence of other metabolites of plant-based food consumed in the diet, acting as possible confounding factors. The development of model foods essentially isogenic and nutritionally identical except that in the type and quantity of plant bioactives to be studied represents an important tool in nutritional studies. The extensive knowledge of the regulation of flavonoid pathway in maize can be exploited to obtain ‘near-isogenic’ model foods, which differ only in the content of specific classes of flavonoids. Being obtainable by breeding strategies, maize model foods can provide functional foods that can be used for both animal feeding studies and human intervention trials for assessing the role of flavonoids or other bioactives in preventing chronic diseases. This review will be focused on recent advances regarding the anthocyanin biosynthesis in maize, the role of anthocyanins from corn in preventing chronic diseases and finally on the breeding activities to

produce maize functional foods with increased anthocyanin content.

Keywords Chronic diseases · Functional food · Maize · Model food · Nutrigenomics

Abbreviations

<i>r1</i>	<i>red color1</i>
<i>b1</i>	<i>booster1</i>
<i>c1</i>	<i>colored aleurone1</i> or <i>colorless1</i>
<i>pl1</i>	<i>purple plant1</i>
<i>pl</i>	<i>pericarp color1</i>
<i>pr1</i>	<i>purple aleurone1</i>
bHLH	Basic helix-loop-helix
CHS	Chalcone synthase
CHI	Chalcone isomerase
F3H	Flavanone 3-hydroxylase
F3'H	Flavanone 3'-hydroxylase
DFR	Dihydroflavonol reductase
FLS	Flavonol synthase
ANS	Anthocyanidin synthase
DHK	Dihydrokaempferol
DHQ	Dihydroquercetin

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Introduction

Maize is an ancient cultivated plant which is diffused nowadays all around the world. Its origin is lost beyond the dawn of time in Central America and a lot of myths arose regarding this subject. In one of these tales, maize was held inside a mountain and the divine intervention made it available to the mankind by opening the mountain with a

thunderbolt. This event also represents an explanation for the different colors of the maize seeds: black represents the most external seeds burned by the thunderbolt, followed by blue, red, yellow and finally white, corresponding to the seeds at the center of the mountain not reached by the thunderbolt (Werner 1997).

After the discovery of America in 1492, small amounts of maize samples arrived first in Spain and then spread all over Europe. Since the first historical report, maize seeds were described as having different colors such as white, red and black (Fig. 1a). Initially the cultivation of this cereal in Europe represented a botanical curiosity, partly due to the incapacity of these plants to produce seeds because of the differences in environmental conditions that is from the short photoperiod and warm climate of the tropical areas of America to the long photoperiod and cold-temperate climate of Europe. Later, new seeds arrived from higher latitudes of the Americas which were able to produce fertile plants and maize started to rapidly spread from Spain to the entire Europe (Fig. 1b) and then all over the world. In the process of adaptation/selection to the new growth conditions in the Old World, but also for cultural reasons, maize lost its colors, with rare exceptions. For example in Italy, where maize was adopted very early, local colored varieties are reported (Brandolini and Brandolini 2009). Nowadays, maize cultivated worldwide has yellow kernels, although the maize plant still retain the ability to weakly pigment different tissues, especially in response to abiotic and biotic stresses. We know that these pigments are mainly anthocyanins (the most abundant of which is cyanidin 3-glucoside), which are water-soluble molecules belonging to the class of flavonoids, secondary metabolites synthesized by a complex metabolic pathway consisting of about 20 biosynthetic genes (Chandler et al. 1989). In plants, flavonoids play important roles in the recruitment of pollinators, male fertility, UV protection and in general protecting from oxidative stresses (reviewed by Winkel-Shirley 2002). The flavonoid pathway is regulated by two types of transcription factors encoded by the *bHLH* and *MYB* gene families. Members of the *bHLH* gene family are *r1* (*red color1*) and *b1* (*booster1*), whereas *c1* (*colored aleurone1*), *pl1* (*purple plant1*) and *p1* (*pericarp color1*) belong to the *MYB* gene family (Chandler et al. 1989; Dooner et al. 1991; Pilu et al. 2003). As previously mentioned, the majority of maize varieties are able to accumulate anthocyanins in different tissues, because they carry (in most cases) all the biosynthetic genes involved in the anthocyanin pathway. In fact, almost all maize landraces, inbred lines and hybrids have some tissues pigmented (e.g. seedling roots under light stimuli, anthers etc.) and generally biotic or abiotic stresses enhance the pigmentation level. Hence, the difference between

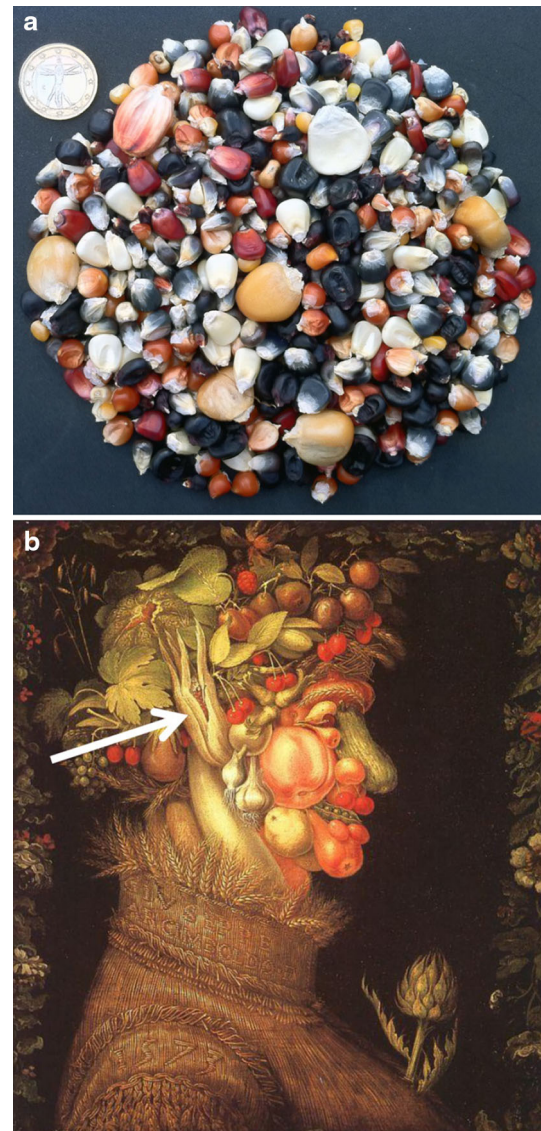


Fig. 1 Biodiversity and diffusion of maize. **a** A survey of maize seeds of different color and shape showing the big genetic variability of this ancient species. On the left, 1 Euro coin. **b** The early diffusion of maize in Europe is also witnessed by the painter Arcimboldo, in one of his most famous masterpieces “The summer” made in 1573. The white arrow indicates a maize ear

“colored” maize varieties and the “uncolored” ones lies in the presence of “strong alleles” of the dominant regulatory genes that up-regulate the biosynthetic genes and determine the anthocyanin pigmentation of specific tissues. These “strong alleles” were early lost, when the first European settlers and the European population started to grow this crop.

In the last years, the scientific community focused its efforts on the study of these pigments, because several papers suggested that foods rich in anthocyanins prevent several chronic diseases, such as cancer, cardiovascular disease, diabetes and obesity (reviewed by Tsuda 2012).

For this reason, breeders and plant biotechnologists are trying to increase the anthocyanin content in several vegetables mainly using three different approaches: rediscovering ancient cultivars, classical breeding and biotechnological approaches. This review will be focused on recent advances regarding the biosynthesis of the anthocyanins in maize, on the effect of these pigments on human health and finally on the breeding activities to produce and increase the anthocyanin content in maize kernels.

The anthocyanin biosynthesis pathway in maize

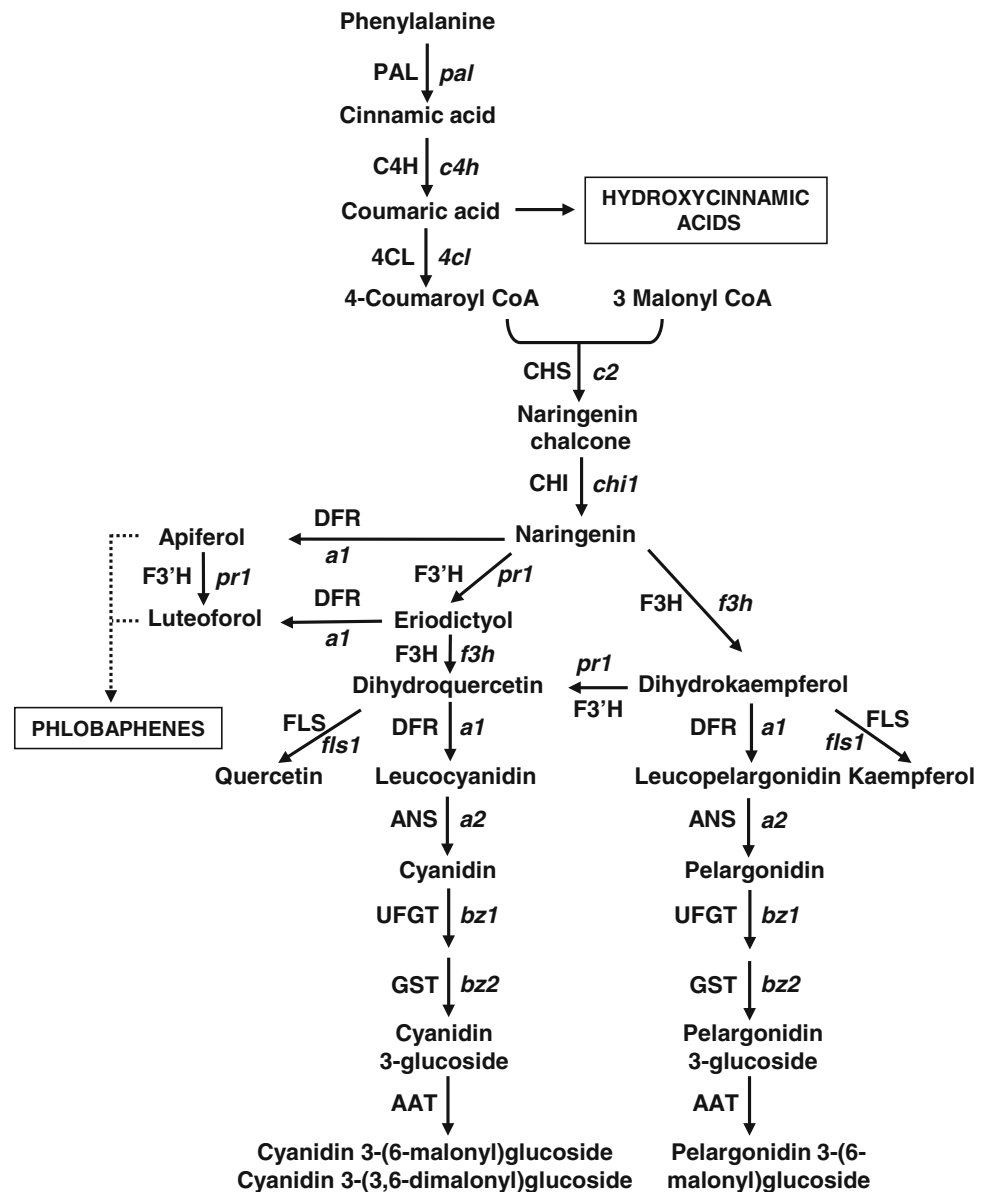
In maize, the anthocyanin biosynthesis stems from the general phenylpropanoid metabolism (Fig. 2; Petroni and Tonelli 2011; Sharma et al. 2012) with the condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA into naringenin chalcone by the chalcone synthase (CHS) enzyme. Naringenin chalcone is then isomerized to naringenin by chalcone isomerase (CHI) and then converted to dihydrokaempferol (DHK), which is the substrate for three different enzymes: (1) dihydroflavonol reductase (DFR) that opens the way to the synthesis of pelargonidin through anthocyanidin synthase (ANS); (2) flavanone 3'-hydroxylase (F3'H) that converts DHK to dihydroquercetin (DHQ) and (3) flavonol synthase (FLS) that catalyzes the formation of kaempferol. Similarly, DHQ is directed to the synthesis of cyanidin by DFR and ANS or to the synthesis of another flavonol (i.e. quercetin) by FLS. Pelargonidin and cyanidin are then glycosylated by UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT), conjugated to glutathione by glutathione S-transferase (GST) and then transferred to the vacuole. Beside cyanidin 3-glucoside and pelargonidin 3-glucoside, a relatively high proportion (about 40 %) of malonylated anthocyanins have been reported in maize, that is cyanidin 3-(6-malonyl)glucoside, cyanidin 3-(3,6-dimalonyl)glucoside and pelargonidin 3-(6-malonyl)glucoside. The dimalonylated cyanidin 3-(3,6-dimalonyl)glucoside, which is one of the main malonylated anthocyanins in maize, has a relatively restricted occurrence in nature and has been also identified in reed canary grass and other grasses (Fossen et al. 2001, 2002; Toufeksian et al. 2008), as well as in chrysanthemum and *Allium victorialis* (Nakayama et al. 1997; Andersen and Fossen 1995). Malonylated anthocyanins are mainly obtained by acyl-CoA-dependent anthocyanin aliphatic acyltransferases (AAT), one of which has been identified in chrysanthemum (Nakayama et al. 2003; Suzuki et al. 2004). In general, malonylation is important to enhance anthocyanin solubility in water, to protect glucosides from enzymatic degradation, to stabilize anthocyanin structures and for the uptake of anthocyanins into vacuoles

(Nakayama et al. 2003). In maize, most anthocyanin biosynthesis genes (encoding CHS, CHI, F3'H, DFR, ANS, UFGT, GST) have been isolated in the past years (Dooner et al. 1991; Petroni and Tonelli 2011), except *pr1*, encoding F3'H, which was only recently cloned and characterized (Sharma et al. 2011). The F3'H enzyme was also recently shown to be involved in the biosynthesis of phlobaphenes, red pigments that are produced by a maize-specific branch of the flavonoid pathway (Sharma et al. 2012). Phlobaphenes are constituted by polymers of apiferol and luteoforol, which are derived from naringenin and eriodictyol, respectively, through the activity of the DFR enzyme. Eriodictyol is also converted to DHQ by the F3'H enzyme (Fig. 2).

Anthocyanins and phlobaphenes are regulated by separate sets of regulatory genes. All anthocyanin biosynthesis genes are coordinately regulated by the *MYB c1/pl1* and the *bHLH r1/b1* gene families. Since each member of these families has a tissue- or development-specific expression, the anthocyanin pigmentation pattern of a maize plant depends on the allelic constitution at the *r1/b1* and *c1/pl1* loci. In fact, *c1* in the seed or *pl1* in plant tissues contribute to the developmental and light-dependent regulation of anthocyanin accumulation (McCarty et al. 1989; Cocciolone and Cone 1993), whereas the *bHLH* genes, such as *R1*, *B1*, *Scutellar node1* (*Sn1*), *Leaf color1* (*Lc1*), *Hopi1*, determine the tissue-specific synthesis of anthocyanins (Tonelli et al. 1991; Goff et al. 1992; Procissi et al. 1997; Petroni et al. 2000). In contrast, phlobaphene synthesis is controlled by the *MYB P1* gene by activating a subset of anthocyanin genes (i.e. CHS, CHI, DFR and FLS) in a limited number of tissues, such as kernel pericarp and cob glumes (Grotewold et al. 1994; Ferreyra et al. 2010). Recent studies have demonstrated that the *pr1* gene, encoding the F3'H enzyme responsible for the conversion of DHK to DHQ (Fig. 2), is also activated by *R1 C1* in aleurone of maize seeds (Sharma et al. 2011) and by *P1* in pericarp of seeds and in cob glumes, where it contributes to the conversion of apiferol to luteoforol, both precursors of phlobaphenes (Sharma et al. 2012).

In addition to the *MYB* and *bHLH* regulators, the *pale aleurone color1* (*pac1*) gene encoding a WD40 protein is required for full activation of anthocyanin biosynthesis genes in seeds (Selinger and Chandler 1999; Carey et al. 2004). In a recently proposed model, bHLH proteins would function as homodimers and as docking proteins between *MYB* and WD40 proteins. Furthermore, the coordinate regulation of anthocyanin biosynthesis genes would be achieved by two different R1/C1 complexes, each able to bind specific *cis*-elements and thus to activate a subset of anthocyanin biosynthesis genes (Kong et al. 2012). In the first complex, R1 homodimerizes via the aspartokinase

Fig. 2 Schematic representation of the flavonoid pathway in maize, comprising the general phenylpropanoid pathway, the anthocyanin and phlobaphene branches. Genes (enzymes) are abbreviated as follows: *pal* (PAL), phenylalanine ammonia lyase; *c4h* (C4H), cinnamic acid 4-hydroxylase; *4cl* (4CL), 4-coumarate CoA ligase; *c2* (CHS), chalcone synthase; *chi1* (CHI), chalcone isomerase; *f3h* (F3H), flavanone 3-hydroxylase; *pr1* (F3'H), flavanone 3'-hydroxylase; *a1* (DFR), dihydroflavonol reductase; *fls1* (FLS), flavonol synthase; *a2* (ANS), anthocyanidin synthase; *bz1* (UFGT), UDP-flavonoid glucosyl transferase; *bz2* (GST), glutathione S-transferase; (AAT), anthocyanin acyltransferase (modified from Petroni and Tonelli 2011; Sharma et al. 2012)



chorismate and TyrA-like (ACT-like) domain, interacts via the bHLH domain with an EMSY-like maize nuclear factor, necessary for histone acetylation of the promoter of anthocyanin biosynthesis genes and interacts via the N-terminal domain with C1, necessary to bind the high affinity P1-binding sites (^{ha}PBS) and low affinity PBS (^{la}PBS) found in the promoters of some anthocyanin biosynthesis genes, such as *a1* (Grotewold et al. 1994; Sainz et al. 1997). In the second complex, R1 homodimerizes via the bHLH domain, directly binds the E-box elements, such as those present in the *bz1* promoter, and interacts via the N-terminal domain with the C1 protein, necessary to provide a strong transcriptional activation domain (Kong et al. 2012).

Corn as model food

The first indication that consumption of flavonoid-rich food promotes health came from the renowned French paradox, which stated that a moderate consumption of red wine, rich in polyphenols such as resveratrol, flavonols, anthocyanins and catechins, was able to decrease the mortality due to coronary heart disease, despite a dairy-rich diet such as the French one (Renaud and de Lorgeril 1992). Since then, many epidemiological studies revealed that consumption of fruit and vegetables in general is associated with a significant reduction of cardiovascular disease, obesity and type 2 diabetes (Humble et al. 1993; Liu et al. 1999; Meyer et al. 2000; McCullough et al. 2002). Recent epidemiological

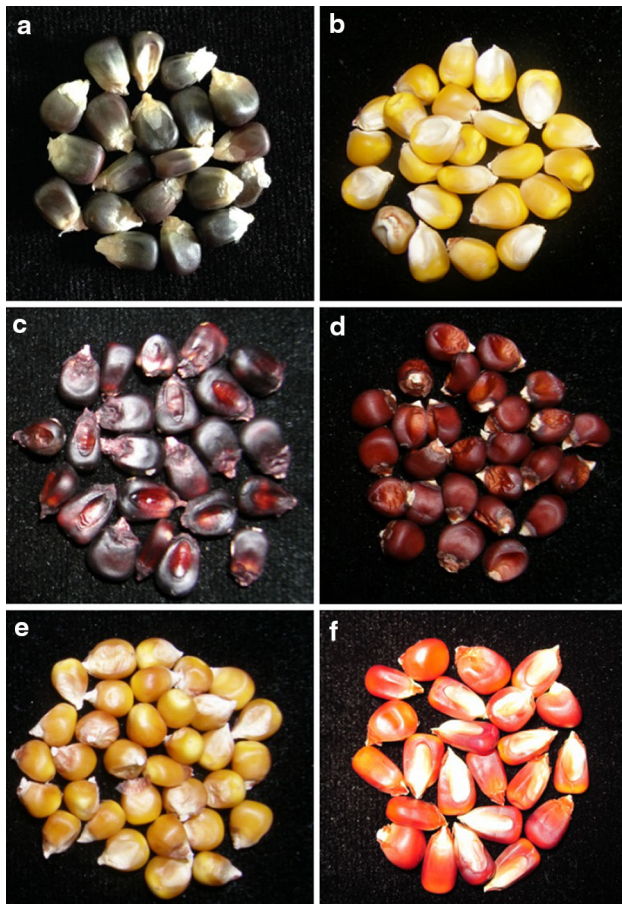


Fig. 3 Near-isogenic maize lines enriched in specific classes of flavonoids. Maize seeds with high levels of anthocyanins in aleurone (a) or pericarp (c). Maize seeds enriched in hydroxycinnamic acids (b), phlobaphenes (d), flavonols (e) and pelargonidin (f)

studies indicated that a high intake of anthocyanin-rich fruits, like blueberry and strawberry, is associated to a reduced risk of myocardial infarction, insulin resistance and inflammation (Cassidy et al. 2013; Jennings et al. 2014).

However, one major limitation in assigning a health property to a specific class of flavonoids in a population-based study is the relative influence of all the constituents of fruits and vegetables consumed in the diet. This can be overcome by comparing near-isogenic plant-based foods that vary only in the type and quantity of the flavonoid class of interest using a nutrigenomic approach in pre-clinical and clinical studies (Martin et al. 2011). The basic idea is to produce model plant materials to test the impact of specific flavonoids on cardiovascular and age-related degenerative diseases in whole foods, since other metabolites may modify their bioavailability and bioactivity. Aim of nutrigenomics is to identify genes and proteins, whose expression is influenced by nutrients, using “omic” tools (transcriptomics, proteomics, metabolomics). Specific

objectives are the understanding of molecular mechanisms underlying nutrient sensing and the identification of molecular biomarkers, useful to identify a preclinical onset of a diet-related disease and to design appropriate dietary recommendations (Muller and Kersten 2003).

The extensive knowledge of the regulation of flavonoid pathway in maize can be exploited to obtain ‘near-isogenic’ model foods which differ only in the content of specific classes of flavonoids (Fig. 3). Using proper combinations of the *MYB* and *bHLH* regulatory genes, near-isogenic lines carrying different levels of anthocyanins and other flavonoids in a desired tissue/organ can be obtained. In maize seed, the dominant *R1 C1* genes activate anthocyanin pigmentation of the aleurone layer in blue corn (Fig. 3a; Procissi et al. 1997), whereas the dominant *B1 P1* genes induce anthocyanin synthesis in the pericarp of purple corn (Fig. 3c; Chandler et al. 1989). Such genes can be further combined to obtain anthocyanin accumulation in multiple tissues within the seed. On the other hand, the anthocyanin content can be modulated using geographic alleles of the *P1* regulatory gene. For example, the pericarp of plants carrying *P1-Rhoades*, a light-independent allele, are deep purple and darker (Fig. 3c) compared to those carrying *p1-bol3* or *p1-W22*, which are partially or completely light-dependent, respectively (Pilu et al. 2003).

Seeds with high levels of phlobaphenes in pericarps can be obtained using the *P1 MYB* regulatory gene (Fig. 3d; Grotewold et al. 1994), whereas maize lines enriched in other flavonoids or metabolic intermediates can be obtained by combining regulatory genes and mutations in specific biosynthetic genes. A mutation in the *A1* biosynthetic gene, encoding DFR, combined with *R1 C1* can be used to obtain flavonol-enriched maize kernels (i.e. quercetin and kaempferol; Fig. 3e), whereas a mutation in the *Pr1* biosynthetic gene, encoding F3'H, combined with *R1 C1* will produce maize kernels enriched in pelargonidin-based anthocyanins and kaempferol (Fig. 3f). When the *pr1* mutation was combined with the *P1* regulatory gene, only apiferol, one of the two precursors of phlobaphenes, was synthesised in pericarps (Sharma et al. 2012). Since all these lines also contain hydroxycinnamic acids in addition to the specific classes of flavonoids, maize plants with recessive alleles of *MYB* and *bHLH* genes containing only hydroxycinnamic acids represents a model food to be used as control in nutritional studies (Fig. 3b).

Health-promoting properties of anthocyanin-rich corn

A clear example of using model foods which are essentially isogenic, except for their content of defined flavonoids, come from nutrigenomic studies on the beneficial effects of dietary anthocyanins from corn against cardiovascular

disease. Maize near-isogenic lines carrying *R1* and *C1* genes, that promote anthocyanin accumulation in aleurone (Procissi et al. 1997), were obtained which differed only by whether they contained or did not contain anthocyanins (Fig. 3a, b; Toufektsian et al. 2008). Such anthocyanin-rich and anthocyanin-free seeds were incorporated in the standard rat diet and used to feed two groups of rats for 8 weeks. The type and levels of anthocyanins in the corn and in the food pellets were determined and their intestinal absorption was verified by measuring anthocyanins in the plasma and urine from rats fed with anthocyanin-rich pellets. These studies demonstrated that in rats fed with anthocyanin-rich maize, the amount of cardiac tissue that was damaged following ischemic conditions was reduced by approximately 30 % compared to rats fed with anthocyanin-free maize (Toufektsian et al. 2008). Cardioprotection was associated with increased myocardial glutathione levels and increased marine omega-3 levels in blood, suggesting that dietary anthocyanins modulate cardiac antioxidant defences and the conversion of plant α -linolenic acid into omega-3 fatty acids (Toufektsian et al. 2008, 2011). The level of dietary anthocyanins in the rodent diet (12 mg/kg body weight per day) was 13 times higher than levels present in an average Western diet. Despite this, the anthocyanin dose tested still represents a physiological dose, that in an average human diet would correspond to a daily intake of 156 mg of anthocyanins, thus being similar to the anthocyanin levels assumed in a typical Mediterranean diet (Wu et al. 2006).

Beside cardioprotection, other studies demonstrated that anthocyanins from purple corn also prevent weight gain and obesity in mice under high fat diet and that they can reduce severe diabetic complications. A purple corn extract normally used as food colorant was incorporated to a standard rodent diet or to a high fat diet and supplied to mice for 12 weeks. The dietary treatment significantly reduced body weight gain and fat accumulation (Tsuda et al. 2003). By impairing adipose tissue accumulation, dietary anthocyanins from purple corn prevented the high *TNF- α* expression level, a pro-inflammatory cytokine normally associated to increase in adipose tissue, which can inhibit pancreatic β -cell function and insulin signaling, resulting in insulin resistance and hyperglycemia. The anti-obesity property of purple corn was likely achieved by reducing the mRNA level of sterol binding protein-1 (SREBP-1), a transcription factor involved in lipogenesis and cholesterol synthesis (Tsuda et al. 2003).

Anthocyanins from purple corn extracts are also effective when supplied in drinking water to diabetic *db* mice for 8 weeks, since they were shown to ameliorate hyperglycemia, insulin sensitivity and to retard diabetic nephropathy, which is one of the most common microvascular complications of diabetes and a leading cause of

end-stage renal disease (Li et al. 2012a). Hyperglycemia is in fact responsible for glomerulosclerosis, excessive blood vessel formation, matrix deposition and consequent altered renal filtration (Zent and Pozzi 2007; Saito et al. 2011). The typical glomerular macrophage infiltration and monocyte activation of kidneys associated to diabetes was inhibited in *db* mice treated with purple corn extracts (Kang et al. 2012) as well as excessive glomerular angiogenesis by reducing the expression of vascular endothelial growth factor (VEGF) (Kang et al. 2013). In vitro studies demonstrated that purple corn extracts can reduce renal mesangial cell proliferation and matrix accumulation, major features of diabetic glomerulosclerosis, and that the most effective anthocyanins are cyanidin 3-glucoside, peonidin 3-glucoside and their acylated derivatives (Li et al. 2012b).

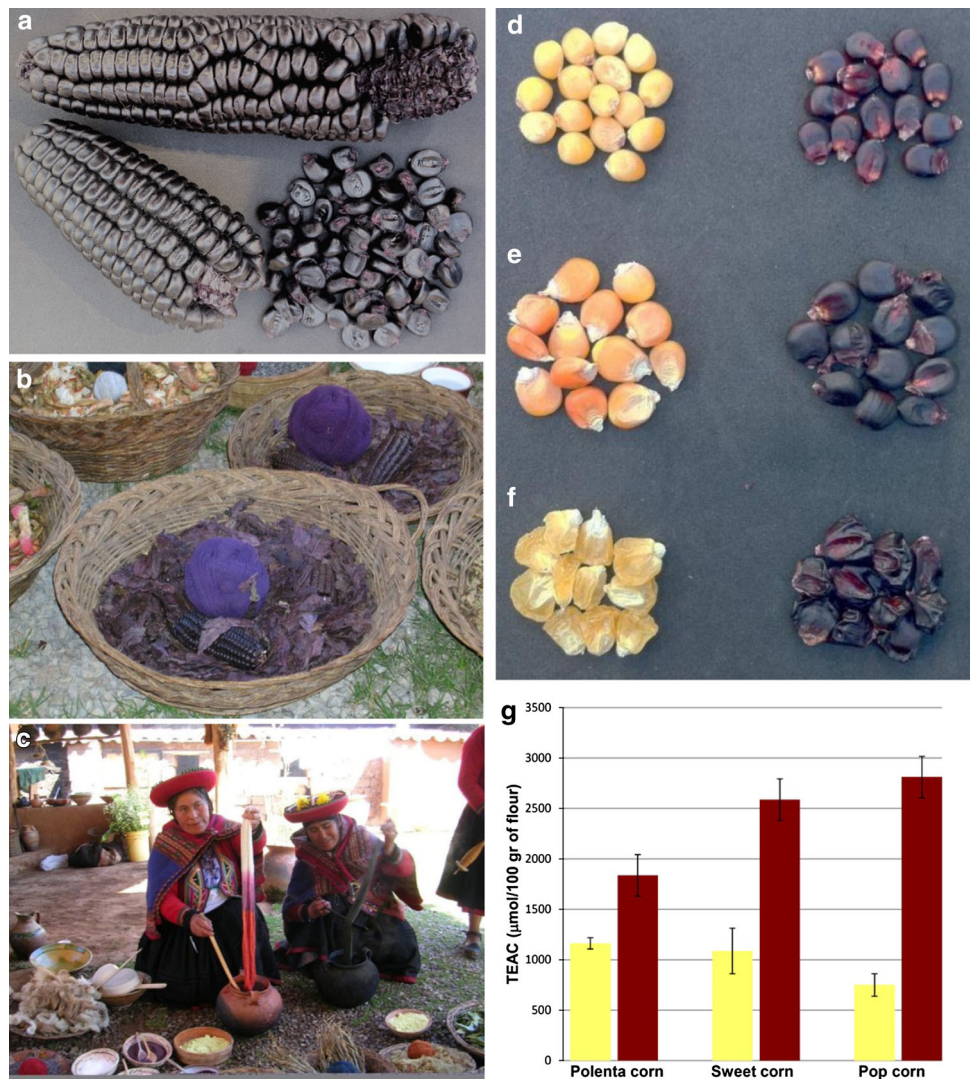
There is some evidence that dietary anthocyanins from purple corn can act as anti-tumor agents in model rats of colorectal and mammary cancer induced by carcinogens, such as heterocyclic amines (Hagiwara et al. 2001; Fukamachi et al. 2008). Recently, purple corn extracts was reported to decrease the incidence of adenocarcinoma in the lateral prostate and to slow down the progression of prostate cancer in a transgenic model rat for adenocarcinoma of prostate. This effect was likely to result from inhibition of proliferation of androgen-dependent prostate cancer cells. The most effective anthocyanins appeared to be both cyanidin 3-glucoside and pelargonidin 3-glucoside (Long et al. 2013).

Anthocyanin-rich corn as functional food

Traditional use for food and beverages in pre-Columbian America

Purple maize named “maíz morado” (Fig. 4a) consists of different open-pollinated varieties traditionally cultivated in South and Central America. The particularity of these varieties is the capacity to accumulate high levels of anthocyanins in the kernel that can overcome the previously reported level of 1600 mg/100 g of dry flour expressed as cyanidin-3-glucoside equivalents (Cevallos-Casals and Cisneros-Zevallos 2003) because of the presence of the *P11-Rhoades* gene (unpublished results). In Peru and Bolivia, purple maize is used as a food to make purple tortillas and the traditional drink “Chicha Morada” (Schwarz et al. 2003), but also as source of natural colorants to be used for tissues staining (Fig. 4b, c; Escribano-Bailon et al. 2004). The “Chicha Morada” is prepared by boiling the maize seeds, usually with pineapple, quince peel, cinnamon and cloves that could be fermented or not (Escribano-Bailón et al. 2004). Traditionally, maize

Fig. 4 Traditional morado and new cultivars developed. **a** Ear and seeds of morado maize cultivar. **b** Balls of wool stained using anthocyanins obtained from morado maize. **c** Tissues staining procedure. Photos taken in Peru by courtesy of Dr. Fabio Francesco Nocito. New cultivars produced by recurrent backcrosses with on the left the colorless recurrent parental for popcorn (**d**), polenta (scagliolo variety, **e**), sweet corn (**f**). **g** Scavenging ability of colored cultivars of polenta corn, sweet corn and popcorn (*in red*) compared to the respective control (*in yellow*) expressed as trolox equivalent antioxidant capacity (TEAC). Confidence intervals at 95 % are shown



tortillas were prepared by introducing whole-kernel in a hot, alkaline solution (usually 1.5–2 % lime, calcium oxide), and subsequently by grinding the drained and rinsed corn (in this form named nixtamal) into a fresh dough called masa that was cooked at low temperature (about 50–60 °C) to produce tortillas (Chapman et al. 2010). Nowadays maize tortillas are also a very common food in these countries, where they provide about 50 % of human energy intake (Villalpando 2004). In Europe this cooking procedure has not been adopted, mainly because the new crop was considered as other well-known cereals (such as wheat, millet, oat and barley). Hence, maize seeds were milled to produce flour using a type of mill that had never been used in pre-Columbian America. Furthermore the “morado” cultivars were lost in Europe and North America, because it was unable to grow and set seeds at those latitudes. When recently planted in northern Italy (45th parallel north), the modern “morado” cultivars also

proved not to be able to produce seeds, because adapted to a growing season with a shorter photoperiod with respect to the European one (unpublished results).

Utilization of corn in human diet in the Western countries

Maize is the main cereal grain cultivated throughout the world, because of its yield and versatility (Food and Agriculture Organization of the United Nations, Crops production 2009; Zeppa et al. 2012). It is used as feed for livestock, as forage, silage and grain, but also as biofuel and for industrial uses. However, human nutrition, that remains one of the main uses, determined the selection of varieties for producing many locally typical cornmeal such as polenta in Italy, angu in Brazil and mush in the USA. Polenta is a very popular dish in the northern regions of Italy (Zeppa et al. 2012). Different maize varieties and

different types of flour milling allowed the production of different kinds of polenta. For example, historically Italian polenta maize was obtained from landraces adapted to the several Italian agroclimatic areas (Brandolini and Brandolini 2009). These landraces used in Italy for human feeding have a flint or semi-flint kernel texture (Brandolini and Brandolini 2009).

The composition of corn flour is well defined: starch (80 %), proteins (10–15 %) and lipids (5 %) (Panzeri et al. 2011). However, only few specific studies have characterized polenta: it is known that polenta can be a good source of iron, phosphorus, and also of carotenoids, mainly provitamin A (Rodriguez-Amaya et al. 2008; Brandolini and Brandolini 2009). Polenta was generally considered a food for the poor people: in nineteenth century, because of wars and famine, polenta was often the only dish consumed by humble and poor people, which led to their getting sick of pellagra, a dietary deficiency of niacin and tryptophan (Sebrell 1981).

Another direct utilization of corn in human diet is the consumption of immature seeds, in particular of sweet cultivars. Sweet corn is different from starchy corn, because it is homozygous for one or more recessive mutations that prevent the polymerization of glucose to starch, causing an accumulation of free sugars in the kernel. At maturity when the seed undergoes dehydration, the seeds wrinkling collapse. Because of these characteristics, sweet corn is used immature, harvested before physiological maturation, during the milk stage, approximately 15–23 days after pollination, when it retains the highest amount of sugar and its maximum sweetness (Hansen R, content specialist, AgMRC, Iowa State University, Sweet corn profile http://www.agmrc.org/commodities_products/grains_oilseeds/corn_grain/sweet-corn-profile/).

There are three different classes of mutations causing this phenotype: *sugary1* (*su1*), *sugary enhancer1* (*se1*), and *shrunk2* (*sh2*). The most ancient and diffused sweet corn is *sugary1*, that is characterized by a texture and flavour traditionally appreciated by consumers (Juvik et al. 2003).

Another important utilization of maize as food is popcorn, an American snack that became one of the favourite snack foods also in a large part of the world (Karababa 2006). Among the snacks, it is one with the best nutritional features: it is constituted by about 78 % of carbohydrates, 13 % of proteins, 4.5 % of fats with also high amounts of iron and calcium (3.2 and 7 mg, respectively, per 100 g) (<http://ndb.nal.usda.gov/ndb/foods>). Without dressing, popcorn has low calories and it is rich in bran (about 14.5 g per 100 g) (Ziegler et al. 1984). There are many other important utilizations of maize in direct human diet, such as corn flakes (popular breakfast invented by Kellogg in 1895 and diffused all over the world), and fermented to produce alcoholic beverages.

Methodologies to produce anthocyanin-rich corn foods

Today, knowing the importance of phytonutrients in the diet to prevent chronic degenerative diseases, a key challenge for maize breeders is to enhance the nutritional value of corn grain. Considering the high popularity of popcorn, polenta and sweet corn in the world and the capacity of corn to accumulate high levels of anthocyanins in the kernel, these foods could become important functional foods able to increase the daily intake of antioxidant compounds. Four strategies can be used to develop new colored varieties, starting from the colorless ones. First, “morado” seeds coming from South America can be directly used for the breeding program. Unfortunately, as previously written, the “morado” cultivars were lost in Europe and North America, because unable to grow at high latitude. However, in particular conditions, in late summer, “morado” plants can be used as pollen donors to pollinate European plants to produce a F1 useful as starting point for the subsequent backcrosses (bc) with the European recurrent parental, selecting in every bc cycle for the colour of kernel and above all for the photoperiod. Second, seeds carrying strong alleles of the regulatory genes of the anthocyanin pathway already adapted to our latitude can be used (e.g. from the USA stock center, <http://www.maizegdb.org/>). Nevertheless, in this case, a genetic background able to drive the anthocyanin accumulation at very high levels have to be reassembled. Third, some European/North American traditional colored varieties, still grown locally as botanical curiosity or for small family production can be used in the breeding program. However, to our knowledge, the level of anthocyanins accumulated in the kernels is not as high as in the cases previously mentioned. This is due to the absence of *P1* gene from these genetic backgrounds that often carries *R1* or *P1* genes instead (unpublished results). Fourth, the last strategy is the utilization of transgenic techniques to directly insert the regulatory genes in the suitable genetic background, able to up-regulate the anthocyanins pathway as reported in tomato by Butelli et al. (2008). In this case, being the final product a genetically modified organism (GMO) utilization in many countries could be very difficult, because of the resistance of the public opinion and governments to the use of GMO foods, as reported in the case of Golden Rice and other crops (Berman et al. 2013).

In the last years, we focused our attention on the second strategy. Using as source of regulatory genes a synthetic variety carrying *B1* and *P1* alleles, we developed popcorn through recurrent backcrosses (Lago et al. 2013), polenta (Lago et al. 2014) and sweet corn (Lago et al. 2014) rich in anthocyanins, that could help to increase the daily intake of these healthy molecules (Fig. 4d, e, f). In fact, although these new cultivars accumulate low levels of anthocyanins

in the kernels with respect to the original “morado”, they possess a higher scavenging ability compared to the respective controls measured as trolox equivalent antioxidant capacity (TEAC) (Fig. 4g) and in some cases also a lower level of mycotoxin contamination (Pilu et al. 2011). Of course, to establish the actual final intake of anthocyanins/antioxidant capacity, the cooking procedure/pre-treatment of the corn in the different utilizations have to be considered, since it is known that the anthocyanin content and stability in the final products are affected by both food processing and temperature (Giusti and Wrolstad 2003). However, preliminary analyses showed that only a 20 % reduction in anthocyanin content occurred after baking cookies in oven (unpublished results).

During the development of these cultivars, an important aspect to keep in consideration was the study of the parameters affecting the final amount of anthocyanins in kernels to plan the breeding work, such as seed weight, pericarp thickness, environmental conditions and the expression level of regulatory genes. Among parameters affecting the anthocyanin concentration, the instability of the *P1* gene expression is a limiting factor, since it is well known that this locus undergoes epigenetic modifications and changes spontaneously to the silenced state with high frequency complicating the breeding work and the maintaining of colored cultivars (Hollick et al. 1995; Pilu 2011).

Concluding remarks

Evidence that consumption of anthocyanin-rich food promotes health is supported by epidemiological studies and preclinical experiments performed with different dietary sources of anthocyanins, showing that they can protect against cancer, cardiovascular disease and age-related neurodegenerative diseases (Tsuda 2012; Martin et al. 2013). However, one major limitation in assigning a health property to anthocyanins or other classes of flavonoids is the influence of other metabolites of fruits and vegetables consumed in the diet, acting as possible confounding factors. The development of model foods essentially isogenic and nutritionally identical except that in the type and quantity of plant bioactives to be studied represents an important tool in preclinical and clinical nutritional studies (Martin et al. 2011). The great advantage of using maize as model food for such studies is that, being obtained by breeding strategies, it can provide non-GMO functional foods that can be used for both animal feeding studies and human intervention trials for assessing the role of flavonoids or other bioactives in the diet. Furthermore, due to the widely available technological infrastructure, corn cultivation can be easily scaled-up, thus representing an easily accessible cost-effective and sustainable food

product. Nutrigenomic studies comparing anthocyanin-rich and anthocyanin-free corn demonstrated that nutritional studies can benefit from plant science and in turn can offer hints to ameliorate crops and obtain plant-based foods with functional properties.

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