UNIVERSITA' DEGLI STUDI DI MILANO

SCUOLA DI DOTTORATO in
Scienze Molecolari e biotecnologie vegetali, alimentari e ambientali

Dipartimento di
Scienze Agrarie e Ambientali – Produzione, Territorio, Agroenergia

CORSO DI DOTTORATO in

Biologia Vegetale e Produttività della Pianta Coltivata

XXVI ciclo

Study of maize genotypes rich in anthocyanins for human and animal nutrition

settore disciplinare: *AGR/07*

Dottorando: Chiara LAGO

Matricola: R09162

Relatore: Dr. Salvatore Roberto PILU

Correlatori: Prof. Gian Attilio SACCHI

Dr.ssa Elena CASSANI

Coordinatore: Prof. Piero Attilio BIANCO

A.A. 2012/2013

Contents

GENERAL INTRODUCTION AND THESIS SUMMARY

Anthocyanin molecules

The flavonoid group of anthocyanins represent the most important secondary metabolites produced by vascular plants: they are responsible for the orange, red, purple, violet and blue colors of flowers, fruits and vegetative parts of the plants. These pigments are present mainly in the form of heterosides in the vacuole of plant cells: a basic structure, called aglycon or anthocyanidin, is bound to a sugar moiety (Clifford, 2000). Two aromatic rings (A and B) and an heterocyclic one containing oxygen (C) form the flavylium ion basic form, conferring them a resonant structure responsible for the anthocyanidin color intensity (Wrolstad *et al.*, 2005). In nature there are 23 anthocyanidins differing by the number and position of the hydroxyl and methoxyl groups in the B ring (Castaneda-Ovando *et al.* 2009): cyanidin (Cy), delphinidin (Dp), malvidin (Mv), pelargonidin (Pg), peonidin (Pn) and petunidin (Pt) are the most represented. Also the sugar moieties can differ in type, number and position, the most diffused are glucose, galactose, arabinose, rhamnose, xylose, and fructose (Delgado-Vergas *et al.*, 2000). The sugar molecule in turn can be acylated with coumaric, caffeic, ferulic, p-hydroxy, benzoic, synaptic, malonic, acetic, succinic, oxailic and malic organic acids that can change in position and number other than in type (Delgado-Vergas *et al.*, 2000). Consequently more than 500 anthocyanins exist (Castaneda-Ovando *et al.*, 2009) widespread in plant kingdom, for which they serve essential functions (Winkel-Shirley, 2002): their chemical structure in fact seems to give them strong antioxidant ability (Prior and Wu, 2006).

Anthocyanin properties

The accumulation of anthocyanin pigments in vegetative tissues is a hallmark of plant stress, even if the clear role is still poorly understood. In many cases, these compounds may provide antioxidant activity as a part of a general stress response (Winkel-Shirley, 2002). Actually there are evidences for their role in radiation protection thanks to their ultra-violet (UV)-absorbing characteristics and their

presence in the epidermal cell layers of leaves and tissues that are susceptible to UV light (Winkel-Shirley, 2002). Moreover they seem able to protect leaf cells from photo-oxidative damage during the leaves senescence of fall time, when many plants display all the anthocyanin colors (Feild *et al.*, 2001) and they also act as phytoalexins in some legumes (Dixon *et al.*, 2001).

Some studies had also associated the anthocyanins and the flavonoids with a resistance to aluminum toxicity in maize (Kidd *et al.*, 2001) and to the control of the polar transport of auxin (Winkel-Shirley, 2002). Regarding maize, Frascaroli and Landi already hypothesized an involvement of the flavonoid pathway with yield (Frascaroli and Landi, 1998) and in our studies we found an association of important agronomic traits correlated to yield with a QTL tightly linked to the *r1* gene, a regulator of anthocyanin biosynthesis.

However the anthocyanin surely play an essential role in plant reproduction not only by conditioning male fertility, but above all by recruiting pollinators and seed dispersers (Winkel-Shirley, 2002). In fact flowers and berries are often rich in these pigments, even if their content can vary not only among different species, but also in the same variety, according to many factors such as temperature, light, genetic background and agronomic treatments (de Pascual-Teresa and Sanchez-Ballesta, 2008). For example blackberries could contain from 83 to 326 mg/100g of pigments, blueberries from 25 to 495 mg/100g (Mazza and Miniati, 1993), and black currants from 130 to 400 mg/100g (Timberlake, 1988). Berries, red wine, purple vegetables and cereals, such as black rice and purple maize are very important anthocyanin-rich sources (Escribano-BailÓn *et al.* 2004) and their spread availability in plant kingdom is so wide that they can easily enter the human diet.

A diet based on plant derived foods had evidenced its own importance in protecting the human and animal health. One of the first of these studies is the Lyon-Diet (Renaud *et al.*, 1992), but also in the following years the role of the consumption of fruits and vegetables rich in phytonutrients and metabolites has been demonstrated fundamental in the fight against chronic diseases. Chronic diseases are non-communicable diseases of long duration and generally slow progression such as cardiovascular diseases, cancers, respiratory diseases, diabetes

and obesity that are becoming the biggest cause of death worldwide. An unhealthy diet seems to be one important risk factor contributing to the onset of these diseases (Noncommunicable diseases country profiles 2011, ISBN 978 92 4 150228 3).

Anthocyanins seem to have a pivotal role in this context: several studies had displayed that a diet rich in these molecules can increase the antioxidant resources, protecting the human health from chronic diseases (Virgili and Marino, 2008). In fact some interesting tests carried on human beings had proven that the consumption of plant foods rich in these pigments seem to lower the LDL-cholesterol levels and the concentration of oxidized LDL (Castilla P *et al.* 2008; Tsuda, 2012), so that the heart disease risk of death is significantly reduced (Rissanen *et al.*, 2003; Tsuda, 2012). In a research paper regarding the heart attack risk, Amnueysit *et al.* (2010) demonstrated that purple corn used to feed broiler chicken decreased their heart weight in comparison with animals fed with yellow corn. Besides purple corn feeding reduced significantly the abdominal fat in broilers (Amnueysit *et al.*, 2010). In fact the beneficial effects could be extended to the fight against obesity because the body fat accumulation and the weight gain in animal models are inhibited too (Peng *et al.* 2011; Seymour *et al.*2009; Titta *et al.* 2010; Tsuda, 2012). A previous study showed also that mice fed with a high fat diet and water with Moro blood orange juice, a rich source of anthocyanins, didn't gain weight. According to the histological analysis, the decrease in lipid accumulation of the mice adipose tissue treated with Moro juice was due to a marked reduction in adipocyte cell size (Titta *et al.* 2010). Besides also blood glucose levels seems to be lowered by consumption of anthocyanin rich foods, according to some animal models (Tsuda, 2008; Prior *et al.* 2008; DeFuria *et al.* 2009; Tsuda, 2012). The positive effects exerted by the anthocyanin pigments can be also widen to the improvement of visual capacity: it seems that anthocyanins can relax the ciliary smooth muscle (Matsumoto *et al.*, 2005) and increase the peripheral blood flow (Iwasaki-Kurashige *et al.*, 2006), so that transient myopia resulted inhibited and the eye fatigue reduced. Moreover the pigment intake seems to improve also dark adaptation (Matsumoto *et al.* 2003; Tsuda, 2012).

Thanks to their antioxidant power it seems that anthocyanins are also able to interfere with the process of carcinogenesis through the inhibition of cyclooxygenase enzymes, to reduce cancer cell proliferation, inhibiting tumor formation (Hou, 2003; Kang *et al*., 2003; Hou *et al*., 2004). Another positive effect is the prevention of the age-related neurodegeneration and cognitive decline (Tsuda, 2012): their abilities to inhibit neuroinflammation (Goyarzu *et al.* 2004; Shukitt-Hale *et al*., 2008; Lau *et al*., 2007; Tsuda, 2012) and to improve cerebral blood flow (Spencer, 2010; Tsuda, 2012) can be the mechanisms through which memory (Krikorian *et al*., 2010; Tsuda, 2012), cognitive and motor performances (Shukitt-Hale *et al*., 2009(a); Shukitt-Hale *et al*., 2009(b); Tsuda, 2012) are improved. Actually the mechanisms thought to be responsible of all these health benefits seems to be the antioxidant power of the anthocyanins but new studies have highlighted some unconsidered functions beyond the radical scavenging (Tsuda, 2012) such as the regulation of the gene expression (Lila, 2004; Juranić and Žižak, 2005; Espín *et al*., 2007) or maybe through the activation of specific detoxification enzymes (Shih, 2007; Titta *et al*., 2010). Some studies suggest also that the primary anthocyanin mechanism of action is the enhancement of the n-3:n-6 PUFA (polyunsaturated fatty acids) ratios, promoting the formation of anti-inflammatory n-3 PUFAs, able to counteract so many and so different chronic diseases (de Lorgeril *et al*., 2008; di Giuseppe *et al*., 2009; Butelli *et al*., 2008; Toufektsian *et al*., 2008; Vauzour *et al*., 2008; Spencer, 2009; Titta *et al*., 2010; Martin *et al*., 2011). Another topic point is the bioavailability of these compounds after dietary intake. Several animal and human studies outlined a complex picture: most of the feeds contain a lot of structurally different anthocyanins, so that the athocyanins derived molecules present in their plasma and urine becomes almost impossible to be identified (McGhie *et al*., 2003; Cho *et al*., 2004; Crozier *et al*., 2010). Besides the absorption and the excretion mechanisms of the pigments seem to be affected by the type of the conjugated sugar molecule and by the structure of the anthocyanidin itself (McGhie *et al*., 2003; Wu *et al*., 2005; Crozier *et al*., 2010), as well as by the source of anthocyanins, the matrix (juice, extract, and capsules) and even by the total amount dosed (Manach *et al*., 2005; Prior and Wu, 2006).

Anthocyanin in maize (*Zea mays* L.)

Given the important role played in human health, anthocyanins are the topic subject of several studies of nutrigenomics, with the aim to identify the genes whose expression is influenced by these specific metabolites. In doing so, plant science can be instrumental, in fact plant breeding can improve varieties in order to boost specific metabolites, but also can develop near-isogenic food, that differ only in the content of a specific phytonutrient, reducing the complexity in the diet-health relationship studies. A suitable example of such an approach is maize (Martin *et al.*, 2011). Maize is able to accumulate anthocyanins, in particular the most abundant anthocyanin is cyanidin-3-glucoside (Escribano-Bailon *et al.*, 2004; de Pascual-Teresa *et al.*, 2002; Nakatani *et al.*, 1979). In fact, after maize has been domesticated in Mexico and then spread into the surrounding regions in the following millenia (Grobman *et al.*, 2012), multicolored, red, purple, blue, and black maize kernels were usually produced, mainly in Peru and Bolivia, long before European settlers arrived (Abdel-Aal *et al.*, 2006; Zĭlić *et al.*, 2012).

So even if maize lost the color in Europe as a result of a selection in behalf of the yellow varieties, corn was originally a pigmented crop.

The pigment biosynthesis begins from a molecule of 4-coumaroyl-CoA and 3 of malonyl-CoA, which are converted by the chalcone synthase (CHS) and chalcone isomerase (CHI) enzymes into naringenin. After hydroxylation into dihydrokaempferol (DHK), the dihydroflavonol reductase (DFR), the flavanone 3'-hydroxylase (F3'H) and the flavonol synthase (FLS) use DHK to synthesize respectively anthocyanins (i.e. pelargonidin), dihydroquercetin (DHQ) and to form flavonols (i.e. kaempferol). Similarly, DHQ can also be converted to the anthocyanin biosynthesis (i.e. cyanidin) by DFR and to flavonol synthesis (i.e. quercetin) by FLS. After the glycosilation and the acylation carried out by UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT) and glutathione S-transferase (GST), pelargonidin and cyanidin are then transferred to the vacuole (Fig.1).

In maize the synthesis of all these enzymes are regulated by different regulatory genes, that can be divided in two families, one encoding MYB transcription factors

(*c1/pl1* family) and one the bHLH transcription factors (*r1/b1* family). To activate all anthocyanins biosynthesis genes in a coordinate manner (Hernandez *et al.*, 2004), one MYB-related protein and one bHLH-containing protein must interact and form a MYB-bHLH heterodimer, with also the need of a WD40 protein in seeds (Carey *et al.*, 2004; Selinger and Chandler, 1999).

The members of each family at the *r1/b1* and *c1/pl1* loci have a tissue or a development specific expression, so that a different pigmentation pattern will be present in the corn plant according to this allelic combination. For example the dominant *B1 Pl1* genes are able to induce anthocyanin synthesis in the pericarp of purple corn, while the dominant *R1 C1* genes are able to activate the pigmentation in the aleurone layer of blue corn (Procissi *et al.*, 1997). By contrast, the *MYB P1* gene, required for phlobaphene synthesis in kernels and cobs, activates a subset of anthocyanin genes (*i.e. CHS, CHI, DFR* and *FLS*) without bHLH interactors. (Grotewold, *et al.*, 1994; Ferreyra *et al.*, 2010).

In the past years the study of anthocyanins biosynthesis and of their regulatory genes revealed some epigenetic phenomena of paramutation involving these MYB and bHLH transcription factors. Specifically an epigenetic phenomenon is "*the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence*" as defined by Riggs and colleagues (Russo *et al.*, 1996), while paramutation occurs when the epigenetic state of an allele called 'paramutagenic' is transferred *in trans* to another allele defined 'paramutable', resulting in a heritable modification of its gene expression. The paramutation can give rise to several epialleles with different silencing degree, able to create variable phenotypes (Chandler, 2007).

One of the first examples of this new kind of gene regulation was found precisely in maize by Brink in 1956. It involved the paramutagenic *R-stippled* (*R-st*) allele and the paramutable *R-r* allele of the *colored1* (*r1*) gene (Brink, 1956). Other examples in maize were discovered in the *b1* and *pl1* loci. The paramutable *B-I* (*Booster-Intense*) and *Pl-Rh* (*Pl-Rhoads*) alleles spontaneously become partially silent (*B'*) and (*Pl'*) alleles with paramutagenic activity (Coe, 1959; Coe, 1966; Hollick *et*

al., 1995). Finally even in the *p1* locus can spontaneously arise the epialle *P-rr'* with a weak paramutagenic ability on *P-rr* (Das *et al.*, 1994; Sidorenko *et al.*, 2001).
All these paramutation systems showed some differences in the epigenetic states stability, higher in *p1* and *r1* systems then in *pl1* and *b1* loci (Chandler *et al.*, 2000; Sidorenko and Peterson, 2001); also the paramutagenicity level can differ among alleles: it's high in *B'* and *Pl'* (Hollick *et al.*, 1995), variable in *R-st* (Kermicle *et al.*, 1995) and *P-rr'* (Sidorenko *et al.*, 2001; Sidorenko and Chandler, 2008). Behind these kind of phenomena, recent findings displayed an involvement of the gene silencing machinery induced by miRNAs and foreign dsRNA (Das and Messing, 1994; Kermicle *et al.*, 1995; Lund *et al.*, 1995; Chandler *et al.*, 2000; Sidorenko and Peterson, 2001; Chandler and Stam, 2004; Stam and Scheid, 2005; Alleman *et al.*, 2006; Chandler, 2007; Pilu *et al.*, 2009). These paramutation phenomena should be kept in consideration in breeding programs: in particular *Pl* silencing forces breeders to select the most colored individuals in each generation.

Summary of the thesis

Anthocyanins are very important nutraceuticals and first of all they are important molecules for the plants: these pigments can protect against biotic and abiotic stresses (Winkel-Shirley, 2002) but they also seem to be involved in the yield (Frascaroli and Landi, 1998). In any case, they exert antioxidant abilities and thanks to this properties, anthocyanin-rich foods exert interesting function to protect human and animal health (Virgili and Marino, 2008). Maize is able to accumulate anthocyanin in different tissues (Escribano-Bailon *et al.*, 2004; de Pascual-Teresa *et al.*, 2002), so that it can be considered a functional food. On this basis the general aim of this PhD project was to develop maize genotypes with the usual commercial and nutritional value and at the same time able to accumulate anthocyanins in kernels, so to confer the new colored varieties a surplus value compared to the uncolored traditional ones. The development of new colored maize lines would be useful also to study the anthocyanin accumulation mechanism and to increase the knowledge about this subject matter.

As mentioned before, different allele combinations of the anthocyanin regulatory genes are able to color different tissues, in different extent. An evaluation of these different genotypes had been performed to find out the best combination for a significant pigment accumulation and at the same time a good fitness in growing. From these analyses interesting results came to light: the *r1* gene seemed to be tightly associated to a QTL involved in maize yield (Pilu *et al.*, 2012, pag.18). This could be useful for genetic improvement and to develop colored maize with good yield. Anyway the *R1/C1* combination lines weren't able to achieve the anthocyanin level in kernels that the *B/Pl* genotypes reached. Therefore these last genotypes were used to generate the lines characterized in this PhD thesis (popcorn, polenta and sweet corn). An important aspect of this thesis was to study the parameters affecting the anthocyanin final amount in kernels to plan the breeding work. The seed weight, the pericarp thickness, the environment and the expression level of the regulatory genes had been considered. From these studies a primary role of epigenetic phenomena emerged; this fact can complicate the breeding work, so surely this aspect must be examined more in depth (Lago *et al.*, 2011, pag. 40). However, three strategies were available to develop new colored varieties: to select colored alleles from the U.S.A. stock center (http://www.maizegdb.org/), to use colored seeds coming from South America and cross them with our plants or to study some Italian traditional colored varieties, still cultivated as local reality. Among the three, we focused our attention on the first one in this project, even if some works regarding the other 2 strategies have been carried on. We thought that pop corn, polenta and sugary corn colored with anthocyanin could be good sources of these nutraceuticals: belonging to the Italian and to the American tradition, these foods can help to increase the daily intake of these healthy compounds (Lago *et al.*, 2013, pag.49; Lago *et al.*, pag. 77; Lago *et al.*, pag 107).

To develop them, a recurrent breeding scheme was used: a commercial yellow line was crossed with the colored variety carrying the *B/Pl* alleles for the anthocyanin biosynthesis. Then the heterozygous individuals chosen through a Marker Assisted Selection (M.A.S) and for higher pigment content were recurrently

backcrossed five times with the respective uncolored line. Finally some cycles of self pollination were performed to freeze the genotypes and to obtain the new colored lines. To take into account is the fact that the popping ability and the polenta characteristics are quantitative traits, while the sugary one is given by a single a single recessive naturally-occurring genetic mutation (Lago *et al.*, 2013, pag.49; Lago *et al.*, pag. 77; Lago *et al.*, pag 107).

The anthocyanin final amount in the kernels of the new developed colored lines has been considered as competitive compared to the usual anthocyanin sources, but some other analysis regarding the cooking effects and the nutraceutical value were performed. In fact it is known that anthocyanin stability in cooked foods is dependent on the temperature and on the heating time of the thermal process (Abdel-Aal *et al.*, 2003; Cabrita *et al.*, 2000; Hiemori *et al.*, 2009). In our results mild cooking seemed not to affect significantly the pigment content, but strong cooking such as the microwave and the autoclave processes were able to degrade them of around the 46% and 80% respectively (Lago *et al.*, 2013, pag.49; Lago *et al.*, pag 107). Consequently to this loss, the antioxidant capacity decreased too, even if it remained higher than that of the uncolored respective line, attesting the fact that the anthocyanin molecules not degraded by the processing did not change their structure or their beneficial ability (Lago *et al.*, 2013, pag.49; Lago *et al.*, pag. 77; Lago *et al.*, pag 107).

Moreover the anthocyanin presence seemed not to affect the appreciability degree tested on a blinded group of subjects, the new color products could be ready to try the market (Lago *et al.*, 2013, pag.49; Lago *et al.*, pag. 77; Lago *et al.*, pag 107).

This kind of work and analyses are very interesting in regard the fact that two Near Isogenic Lines (NIL), differing for the synthesis of only one class of metabolites, are under comparison. This fact can simplify the study of the advantage given by the presence of the anthocyanins in foods; moreover these isogenic foods can also be used to analyze the relationship between the presence of the pigments in foods and the human and animal health and consequently to confer a property to a specific single class of molecules, simplifying the analyses and the reading of results.

Figures

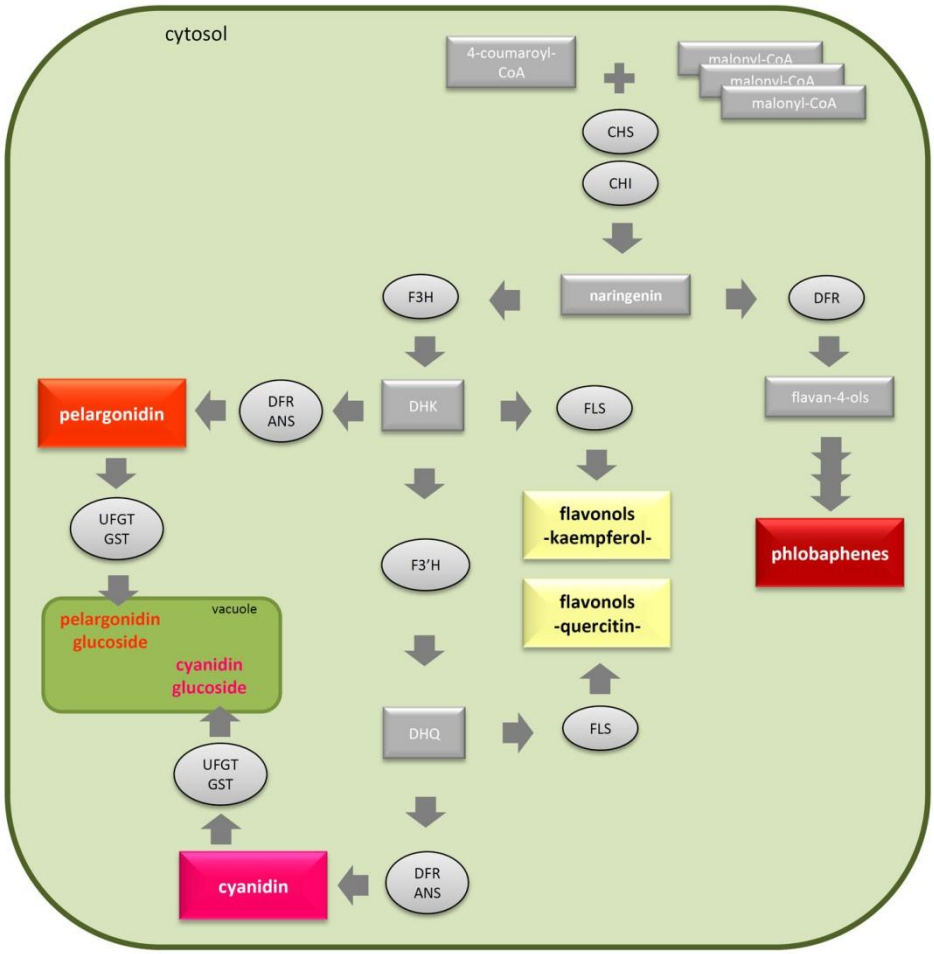


Figure 1. Schematic and simplyfied pathway of the anthocyanin biosynthesis in maize. CHS=chalcone syntase; CHI=chalcone isomerase; DHK=dihydrokaempferol; F3'H=flavanone-3'-hydroxylase; FLS=flavonol synthase; DHQ=dihydroquercitin; DFR=dihydroflavonol reductase; ANS=anthocyanidin synthase; UFGT=UDR glucose flavonoid 3-O-glucosyltransferase; GST=glutathione S-transferase.

References

Abdel-Aal, E.-S. M.; Hucl, P. Composition and stability of anthocyanins in blue-grained wheat. *J. Agric. Food Chem.* **2003**, *51*, 2174–2180.

Abdel-Aal E-SM, Young JC, Rabalski I (2006). Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *J Agric Food Chem* **54**:4696−4704.

Alleman M, Sidorenko L, McGinnis K, Seshadri V, Dorweiler JE, White J, et al. (2006). An RNA-dependent RNA polymerase is required for paramutation in maize. *Nature* **442**(7100):295-8.

Amnueysit P, Tatakul T, Chalermsan N, Amnueysit K (2010). Effects of purple field corn anthocyanins on broiler heart weight. *As J Food Ag-Ind* **3**(03): 319-327.

Brink RA (1956). A Genetic Change Associated with the *R* Locus in Maize Which Is Directed and Potentially Reversible. *Genetics* **41**(6):872-89.

Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EGWM, Hall RD, Bovy AG, Luo J, Martin C (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nat Biotechnol* **26**: 1301–1308.

Cabrita, L.; Fossen, T.; Andersen, O. M. Color and stability of the six common anthocyanidin 3-glucosides in aqueous solution. *Food Chem.* **2000**, *68*, 101–107.

Carey CC, Strahle JT, Selinger DA, Chandler VL (2004). Mutations in the *pale aleurone color1* regulatory gene of the *Zea mays* anthocyanin pathway have distinct phenotypes relative to the functionally similar *TRANSPARENT TESTA GLABRA1* gene in *Arabidopsis thaliana*. *Plant Cell* **16**(2):450-64.

Castaneda-Ovando, Pacheco-Hernandez MdL, Paez-Hernandez ME, Rodriguez JA, Galan-Vidal CA (2009). Chemical studies of anthocyanins: a review. *Food chemistry* **113**:859-871.

Castilla P, Dávalos A, Teruel JL, Cerrato F et al. (2008). Comparative effects of dietary supplementation with red grape juice and vitamin E on production of superoxide by circulating neutrophil NADPH oxidase in hemodialysis patients. *Am J Clin Nutr* **87**:1053–1061.

Chandler VL, Eggleston WB, Dorweiler JE (2000). Paramutation in maize. *Plant Mol Biol* **43**(2-3):121-45.

Chandler VL (2007). Paramutation: from maize to mice. *Cell* **128**(4):641-5.

Chandler VL, Stam M (2004). Chromatin conversations: mechanisms and implications of paramutation. *Nat Rev Genet* **5**(7):532-44.

Cho MJ, Howard LR, Prior RL, Clark JR (2004). Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry. *J Sci Food Agric* **84:**1771–1782.

Clifford MN (2000). Anthocyanins – nature, occurrence and dietary burden. *J Sci Food Agric* **80**:1063-1072.

Coe EH (1959). A Regular and Continuing Conversion-Type Phenomenon at the B Locus in Maize. *Proc Natl Acad Sci U S A*. **45**(6):828-32.

Coe EH (1966). The properties, origin, and mechanism of conversion-type inheritance at the B locus in maize. Genetics **53**(6):1035-63.

Crozier A, Del Rio D, Clifford MN (2010). Bioavailability of dietary flavonoids and phenolic compounds. *Molecular Aspects of Medicine* **31**:446–467.

Das OP, Messing J (1994). Variegated phenotype and developmental methylation changes of a maize allele originating from epimutation. *Genetics* **136**(3):1121-41.

de Pascual-Teresa S, Santos-Buelga C, Rivas-Gonzalo JC (2002) LC-MS analysis of anthocyanins from purple corn cob. *J Sci Food Agric* **82**:1003-1006.

de Pascual-Teresa S, Sanchez-Ballesta MT (2008) Anthocyanins: from plant to health. *Phytochem Rev* **7**:281-299.

de Lorgeril, M., Salen, P., Martin, J.L., Boucher, F., and de Leiris, J. (2008). Interactions of wine drinking with omega-3 fatty acids in patients with coronary heart disease: A fish-like effect of moderate wine drinking. *Am. Heart J.* **155**:175–181.

DeFuria J, Bennett G, Strissel KJ, Perfield JW 2nd et al (2009) Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae. *J Nutr* **139**:1510–1516.

Delgado-Vargas F, Jimenez AR, Paredes-Lopez O (2000). Natural pigments: Carotenoids, anthocyanins and bethalains – Characteristics, biosynthesis, processing and stability. Critical reviews in food science and nutrition **40**(3):173-289.

di Giuseppe R et al., European Collaborative Group of the IMMIDIET Project (2009). Alcohol consumption and n-3 polyunsaturated fatty acids in healthy men and women from 3 European populations. *Am J Clin Nutr* **89**:354–362.

Dixon RA (2001). Natural products and plant disease resistance. *Nature* **411**:843-847.

Escribano-Bailón MT, Santos-Buelga C, Rivas-Gonzalo JC (2004) Anthocyanins in cereals. *J Chromatogr A* **1054**:129-141.

Espín JC, García-Conesa MT, Tomás-Barberán FA (2007). Nutraceuticals: Facts and fiction *Phytochemistry* **68**:2986–3008.

Feild TS, Lee DW, Holbrook NM (2001). Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. *Plant Physiol* **127**:566-574.

Ferreyra ML, Rius S, Emiliani J, Pourcel L, Feller A, Morohashi K, et al. (2010) Cloning and characterization of a UV-B-inducible maize flavonol synthase. *Plant J* **62**(1):77-91.

Frascaroli E, Landi P (1998) Allelic frequency change of *P1* gene in a maize population after recurrent selection for grain yield. *Crop Sci* **38**:1391-1394.

Goyarzu P, Malin DH, Lau FC, Taglialatela G et al. (2004). Blueberry supplemented diet: effects on object recognition memory and nuclear factor-kappa B levels in aged rats. *Nutr Neurosci* **7**:75–83.

Grobman A, Bonavia D, Dillehay TD., Piperno DR, Iriarte J, Holst I (2012). Preceramic maize from Paredones and Huaca Prieta, Peru. *PNAS* **109**(5):1755-1759.

Grotewold E, Drummond BJ, Bowen B, Peterson T (1994). The myb-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. *Cell* **76**(3):543-53.

Hernandez JM, Heine GF, Irani NG, Feller A, Kim MG, Matulnik T, et al. (2004). Different mechanisms participate in the *R*-dependent activity of the R2R3 MYB transcription factor *C1*. J Biol Chem **279**(46):48205-13.

Hiemori M, Koh E, Mitchell AE (2009). Influence of Cooking on Anthocyanins in Black Rice (*Oryza sativa* L. *japonica* var. SBR). *J Agric Food Chem* 57 (5):1908-14.

Hollick JB, Patterson GI, Coe EH Jr., Cone KC, Chandler VL (1995). Allelic interactions heritably alter the activity of a metastable maize *pl* allele. *Genetics* **141**(2):709-19.

Hou DX (2003). Potential mechanisms of cancer chemoprevention by anthocyanins. *Curr Mol Med* **3**(2):149–159.

Hou DX, Kai K, Li JJ et al. (2004). Anthocyanidins inhibit activator protein 1 activity and cell transformation: structure-activity relationship and molecular mechanisms. *Carcinogenesis* **25**(1):29–36.

Iwasaki-Kurashige K, Loyaga-Rendon RY, Matsumoto H, Tokunaga T et al. (2006). Possible mediators involved in decreasing peripheral vascular resistance wit blackcurrant concentrate (BC) in hind-limb perfusion model of the rat. *Vascul Pharmacol* **44**:215–223.

Juranic Z, Žižak Z (2005). Biological activities of berries: from antioxidant capacity to anti-cancer effects. *Biofactors* **23:**207–211.

Kang S, Seeram N, Nair M, Bourquin L (2003). Tart cherry anthocyanins inhibit tumor development in Apc(Min) mice and reduce proliferation of human colon cancer cells. *Cancer Lett* **194**(1):13–19.

Kermicle JL, Eggleston WB, Alleman M (1995). Organization of paramutagenicity in *R-stippled* maize. *Genetics* **141**(1):361-72.

Kidd PS, Llugany M, Poschenrieder C, Gunsé B, Barceló J (2001). The role of root exudates in aluminium resistance and silicon-induced amelioration of aluminium toxicity in three variety of maize (*Zea mays* L.). *J Exp Bot* **52**:1339-1352.

Krikorian R, Shidler MD, Nash TA, Kalt W et al. (2010). Blueberry supplementation improves memory in older adults. *J Agric Food Chem* **58**:3996–4000.

Lau FC, Bielinski DF, Joseph JA (2007). Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated BV2 microglia. *J Neurosci Res* **85**:1010–1017.

Lila MA (2004). Anthocyanins and human health: an in vitro investigative approach. *J Biomed Biotechnol* **5**:306–313.

Lund G, Das OP, Messing J (1995). Tissue-Specific Dnase-I-Sensitive Sites of the Maize *P*-Gene and Their Changes Upon Epimutation. *Plant Journal* **7**(5):797-807.

Manach C, Williamson G, Morand C, Scalbert A, Remesy C (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* **81**(1 Suppl.):230S–242S.

Martin C, Butelli E, Petroni K, Tonelli C (2011). How Can Research on Plants Contribute to Promoting Human Health? *The Plant Cell* **23**:1685–1699.

Matsumoto H, Nakamura Y, Tachibanaki S, Kawamura S et al. (2003). Stimulatory effect of cyanidin 3-glycosides on the regeneration of rhodopsin. *J Agric Food Chem* **51**:3560–3563.

Matsumoto H, Kamm KE, Stull JT, Azuma H (2005). Delphinidin-3-rutinoside relaxes the bovine ciliary smooth muscle trough activation of ETB receptor and NO/cGMP pathway. *Exp Eye Res* **80**:313–322.

Mazza G and Miniati E (1993). Anthocyanins in fruits, vegetables, and grains. CRC Press, Boca Raton, FL.

McGhie TK, Ainge GD, Barnett LE, Cooney JM, Jensen JD (2003). Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. *J Agric Food Chem* **51:**4539–4548.

Mo Y, Nagel C, Taylor LP (1992). Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. *Proc Natl Acad Sci USA* **89**:7213–7217.

Nakatani N, Fukuda H, Fuwa H (1979). Major anthocyanin of Bolivian purple corn (*Zea mays L.*). *Agric. Biol. Chem*., *43*, 389-391.

Noncommunicable diseases country profiles 2011, ISBN 978 92 4 150228 3.

Peng CH, Liu LK, Chuang CM, Chyau CC et al. (2011). Mulberry water extracts possess an anti-obesity effect and ability to inhibit hepatic lipogenesis and promote lipolysis. *J Agric Food Chem* **59**:2663–2671.

Pilu R, Panzeri D, Cassani E, Cerino Badone F, Landoni M, Nielsen E (2009). A paramutation phenomenon is involved in the genetics of maize *low phytic acid1-241* (*lpa1-241*) trait. *Heredity* **102**(3):236-45.

Prior RL, Wu X (2006) Anthocyanins: Structural characteristics that result in unique metabolic patterns and biological activities. Free Radical Research **40**(10): 1014–1028.

Prior RL, Wu X, Gu L, Hager TJ et al. (2008). Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity. *J Agric Food Chem* **56**:647–653.

Procissi A, Dolfini S, Ronchi A, Tonelli C (1997). Light-dependent spatial and temporal expression of pigment regulatory genes in developing maize seeds. *The Plant Cell* **9**(9):1547-57.

Renaud S, de Lorgeril M (1992). Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **339**(8808):1523-6.

Rissanen TH, Voutilainen S, Virtanen JK, Venho B et al. (2003). Low intake of fruits, berries and vegetables is associated with excess mortality in men: the Kuopio Ischaemic Heart Disease Risk Factor (KIHD) Study. *J Nutr* **133:**199–204.

Russo VE et al. (1996). Epigenetic mechanisms of gene regulation. Cold Spring Harbor Laboratory Press, editor. Woodbury, USA.

Selinger DA, Chandler VL (1999). A mutation in the pale aleurone color1 gene identifies a novel regulator of the maize anthocyanin pathway. *Plant Cell* **11**(1):5-14.

Seymour EM, Lewis SK, Urcuyo-Llanes DE, Tanone II et al. (2009). Regular tart cherry intake alters abdominal adiposity, adipose gene transcription, and inflammation in obesity-prone rats fed a high fat diet. *J Med Food* **12**:935–942.

Shih PH ,Yeh CT, Yen GC (2007). Anthocyanins induce the activation of phase II enzymes through the antioxidant response element pathway against oxidative stress-induced apoptosis. *J Agric Food Chem* **55**:9427–9435.

Shukitt-Hale B, Lau FC, Carey AN, Galli RL et al. (2008). Blueberry polyphenols attenuate kainic acid-induced decrements in cognition and alter inflammatory gene expression in rat hippocampus. *Nutr Neurosci.* **11**:172–182.

(a) Shukitt-Hale B, Cheng V, Joseph JA (2009). Effects of blackberries on motor and cognitive function in aged rats. *Nutr Neurosci* **12**:135–140.

(b) Shukitt-Hale B, Kalt W, Carey AN, Vinqvist-Tymchuk M et al. (2009). Plum juice, but not dried plum powder, is effective in mitigating cognitive deficits in aged rats. *Nutrition* **25**:567–573.

Sidorenko LV, Peterson T (2001). Transgene-induced silencing identifies sequences involved in the establishment of paramutation of the maize *p1* gene. *Plant Cell* **13**(2):319-35.

Sidorenko L, Chandler V (2008). RNA-dependent RNA polymerase is required for enhancer-mediated transcriptional silencing associated with paramutation at the maize *p1* gene. *Genetics* **180**(4):1983-93.

Spencer JPE (2009). Flavonoids and brain health: Multiple effects underpinned by common mechanisms. *Genes Nutr* **4**:243–250.

Spencer JP (2010). The impact of fruit flavonoids on memory and cognition. *Br J Nutr* **104**:S40–S47.

Stam M, Scheid OM (2005). Paramutation: an encounter leaving a lasting impression. *Trends Plant Sci* **10**(6):283-90.

Timberlake CF (1988). The biological properties of anthocyanin compounds. *NATCOL Q Bull* **1**:4–15.

Titta L, Trinei M, Stendardo M, Berniakovich I et al. (2010). Blood orange juice inhibits fat accumulation in mice. *Int J Obes* **34**:578–588.

Tsuda T (2008). Regulation of adipocyte function by anthocyanins; possibility of preventing the metabolic syndrome. *J Agric Food Chem* **56**:642–646.

Tsuda T (2012). Dietary anthocyanin-rich plants: Biochemical basis and recent progress in health benefits studies *Mol Nutr Food Res* **56**:159–170.

Toufektsian MC et al. (2008). Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. *J Nutr* **138**: 747–752.

Virgili F, Marino M (2008). Regulation of cellular signals from nutritional molecules: A specific role for phytochemicals, beyond antioxidant activity. *Free Radic Biol Med* **45**:1205-1216.

Vauzour D, Vafeiadou K, Rodriguez-Mateos A, Rendeiro C, Spencer JPE (2008). The neuroprotective potential of flavonoids: A multiplicity of effects. *Genes Nutr* **3**:115–126.

Wrolstad RE, Durst RW, Lee J (2005). Tracking color and pigment changes in anthocyanin products. *Trends Food Sci Tech* **16**:423–428.

Winkel-Shirley B (2002). Biosynthesis of flavonoids and effects of stress. *Current Opinion in Plant Biology* **5**:218–223.

Wu X, Pittman HE, McKay S, Prior RL (2005). Aglycones and sugar moieties alter anthocyanin absorption and metabolism after berry consumption in weanling pigs. *J Nutr* **135:**2417–2424.

Zĭlić S, Serpen A, Akıllıoğlu G, Gökmen V, Vančetovic J (2012). Phenolic Compounds, Carotenoids, Anthocyanins, and Antioxidant Capacity of Colored Maize (*Zea mays* L.) Kernels. *J Agric Food Chem* **60:**1224−1231.

A QTL involved in maize yield is tightly associated to the *r1* gene on the long arm of chromosome 10

[1]Roberto Pilu, [1]Andrea Bucci, [1]Laura Casella, [1]Chiara Lago, [1]Francesco Cerino Badone, [1]Elena Cassani, [2]Michela Landoni.

[1] Dipartimento di Produzione Vegetale, Università degli Studi di Milano,
Via Celoria 2, 20133 Milano, Italy.
[2] Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy.

This is a pre-copy-editing, author-produced of an article accepted for publication in MOLECULAR BREEDING *following peer review.*

Pilu R, Bucci A, Casella L, Lago C, Cerino Badone F, Cassani E, Landoni M. (2012). A QTL involved in maize yield is tightly associated to the *r1* gene on the long arm of chromosome 10. *Mol Breeding* **30**:799–807.

The definitive publisher-authenticated version is available online at:
http://link.springer.com/article/10.1007/s11032-011-9664-9#page-1

Abstract

We produced and studied for three years two synthetic populations of maize differing in their constitution only for the selected alleles present at the *red color 1* (*r1*) locus (*R-sc vs r-r*). *r1* is a regulatory gene conferring anthocyanin pigmentation in different tissues: the *R-sc* allele confers pigmentation only in the aleurone seed layer, while the *r-r* allele confers pigmentation in several tissues such as root, silk and anther but the seed is colourless. The colourless population (*r-r/r-r*) was characterized by improved agronomic features such as ear weight and plant height compared with the *R-sc/R-sc* coloured population. This finding was confirmed studying single F_4 *R/r* families where the presence of the *r-r* allele conferred positive features, acting as a dominant trait.

Quantitative trait locus (QTLs) analysis performed using molecular markers on the long arm of chromosome 10 (bin 10.06) where the *r1* gene maps, identified a QTL map position for plant height tightly associated to the *r1* gene. Thus the *r1* gene may represent a major QTL or it could be closely linked to another gene involved in the agronomic performance of the two populations studied.

Key words: *Zea mays*, *red color 1*, anthocyanins, QTL analysis

Introduction

A majority of the phenotypic variation in crops is determined by many quantitative genetic traits that were studied, in the past, using mathematical statistics (Mather 1941). However, this methodology cannot help us in the identification and localization of single QTL (Quantitative Trait Locus). With the coming of the molecular era it is now possible to identify the map position of the single genetic components, using molecular markers (Stuber et al. 1987; Paterson et al. 1988; Tuberosa and Salvi 2006). During recent years a lot of genetic maps of the major cultivated plants have been created with the aim of dissecting the genetic bases of important quantitative traits useful in breeding programmes. In maize more than 2000 QTLs have been identified (Lawrence et al. 2008); on the other hand, a lot of these QTLs have not yet been confirmed in different experiments which considered other genetic backgrounds or environments (Campos et al. 2004). Furthermore in the majority of the QTL studies the parents used to create the segregant progeny are selected on the basis of the biggest difference for the character studied, not by considering their general agronomic value. This approach allowed the identification of important QTLs in an efficient way, but it does not pledge a real genetic improvement when the best QTL is introgressed in the elite cultivars (Tuberosa and Salvi 2006). Another important aspect of QTL analysis is the choice of segregant populations such as F_2, backcross (BC), recombinant inbred lines (RIL), doubled haploids (DH), that can influence the QTLs identification and effect (Long et al. 2008). In some cases comparative genomics and synteny allowed us to identify candidate genes responsible for the QTL trait (Chardon et al. 2004). In particular, in maize, in accordance with Robertson's hypothesis (Robertson 1985) theorizing that major mutants (generally qualitative traits) are null/near null alleles at a QTL trait, some important successes have been achieved in QTL mendelization. For example *brachytic 2* mutation (Multani et al. 2003; Pilu et al. 2007) and *dwarf 8* (Peng et al. 1999) which have strong effects on plant height, map in the same regions (respectively on the chromosome regions 1S and 5L) where QTL analysis for this trait has identified the major genes (Beavis et al. 1991). Nevertheless in maize this approach is a

difficult undertaking because 1 cM in the genetic map corresponds to about 2.5 million base pairs and the QTL study may produce inaccurate map positions depending on the population size considered. For this reason, usually a goal of QTL mapping is the utilization of the genomic region isolated by marker-assisted selection (MAS) to develop elite material, as reported for example in the work of Szalma and colleagues (Szalma et al. 2007) and by Bouchez and colleagues (Bouchez et al. 2002).

In this work we observed in two synthetic populations, selected for seed colour, a difference in important agronomic traits, so we conjectured the presence of a major QTL associated to the *r1* gene (conferring the accumulation of anthocyanins in several plant tissues) or a direct effect of presence/absence of these pigments. Anthocyanins are a class of water-soluble molecules (secondary metabolites) produced only in plants and conferring a red-blue colour depending by the vacuole pH where they are stored in glycosylated form (de Vlaming et al. 1983). These substances play important roles in several physiological processes such as UV protection, male fertility, antimicrobial activity and in general they are involved in protection against oxidative damage (reviewed by Winkel-Shirley 2002). In maize, anthocyanins are synthesized by a pathway made up by about 20 genes activated by transcription factors belonging to two gene families: *c1/pl1/p1* (MYB genes) and *r1/b1* (bHLH genes) (Chandler et al. 1989; Dooner et al. 1991; Consonni et al. 1997; Pilu et al. 2003). Anthocyanin accumulation in a specific tissue requires the presence of a member of both families: from the MYB family, *Pl* is required for pigment accumulation in the plant and *C1* acts in the aleurone while *r1/b1* acts in the seed and plant tissues depending on the nature of the alleles present (Roth et al. 1991).

In 1997 Nguetta and Cross found a correlated response in important agronomic traits in maize synthetic populations selected for *R-nj,* an allele of *r1* gene, also Frascaroli and Landi in 1998 found after selection for yield a frequency change in the *P1* gene locus (leading to the synthesis of phlobaphenes in the pericarp conferring a red seed colour) so it seems that in some way the flavonoid pathway might be associated with some important agronomic traits. To further investigate

this phenomenon we studied for three years two synthetic populations (*r/r* vs *R/R*) and single segregant selfed families (*R/r*) with the aim to map the quantitative trait responsible for the positive agronomic performances observed and we found that this QTL is tightly associated to the *r1* gene, nevertheless eventually discarding the hypothesis that the *r1* locus directly represents the gene responsible for this QTL.

Material and Method

Plant material

The origin, phenotype and structural characteristics of the *R1* alleles used in this study have been described in Dooner and Kermicle (1974). *R-sc* (self-coloured aleurone) confers coloured aleurone and green plant (Kermicle 1984). The source of *R-sc* was in the W22 background homozygous dominant for the *a1*, *a2*, *c1*, *c2*, *bz1*, *bz2* alleles and homozygous recessive for the *pl1* and *b1* genes, whilst the source of *r-r* allele was the Dekalb 300 hybrid. The *r-r* allele confers coloured root, anther and silk while the seed is colourless. To simplify the nomenclature in the text the *R-sc* and *r-r* alleles will be referred to respectively as *R* and *r*. The distinctive tissue-specific expression of the *R* alleles used in this study allowed us to easily discriminate the three genotypes *R/R*, *R/r* and *r/r* (Supplementary Material Table 1). We used also the *R-g* allele conferring coloured aleurone and green plant; *r-Δ902* allele (in the text referred as Δ), carrying a deletion involving the *r1* locus, conferring colorless plant and seed tissues (Alleman and Kermicle 1993; Consonni et al. 1997) and the *Sn* locus: a factor lying two map units distal to *r1,* conferring a strong coloration to the seedling tissues (Pilu et al. 2003). All these alleles were in the *W22* background. We used also the *W64A* inbred line as a source of the *r* allele to study the F_2 progeny obtained by selfing the *R/r* plants.

Development of synthetic populations and families

Two synthetic populations were obtained by crossing the colourless F_1 *r/r* Dekalb 300 hybrid with coloured inbred lines W22 homozygous for the *R allele*. The F1

obtained was selfed and about 3000 F2 colored seeds (*R/r* and *R/R*), were selected and sown, the plants with red anthers (*R/r*) were discarded whilst the plants having green anthers (*R/R*) were selfed, obtaining the coloured population homozygous for *R*. We also selected and sowed from the F_2 population, about 1200 colourless seeds (*r/r*) to constitute the colourless control population. The two populations were tested in the 2006, 2007 and 2008 field seasons: 1200 seeds were sown in adjacent separated plots of 140 square meters, under the same agronomic conditions (apart from the 2007 field season where we fertilized the soil using 240 kg/ha of nitrogen). Single *R/r* families were obtained by selfing the plants having coloured seeds and red anthers until they reached the F_4 generation, the object of this study (Supplementary Material Fig. 1). The ears were hand harvested at maturity at the same time, dried to a moisture content of 15 – 16 % using a dryer machine and shelled to determine the ear weight (seed weight for ear). All the field experiments were carried out in the experimental field of the University of Milan located in Landriano (PV).

Seed germination

Seeds were germinated in Plexiglas boxes on filter paper imbibed with water. Seedlings were then grown under a light source consisting of cool white (F36T12/CW/HO) fluorescent lamps from GTE SYLVANIA (Lighting Products Group, Danvers, MA) for 10 days at 25°C and then weighed.

r1 mapping

r1 mapping was carried out using a segregating F_2 population obtained by selfing *R/r* heterozygous plants. We extracted DNA from a piece of leaf of each plant as previously described (Dellaporta et al. 1983). We used *bnlg1028* SSR molecular marker (5'AGGAAACGAACACAGCAGCT3'/ 5'TGCATAGACAAAACCGACGT3') tightly associated to *r1* gene located on the long arm of chromosome 10 from MaizeGDB (http://www.maizegdb.org/ssr.php). Polymerase chain reactions (PCR) and gel

running conditions were performed as described in the SSR Methods Manual by MaizeGDB (http://www.maizegdb.org/documentation/maizemap/ssr_protocols.php). Recombinant values were converted to map distances using MAPMAKER 3 (Lander et al. 1987).

QTL analysis of long arm of chromosome 10

For QTL analysis we used a segregating F_2 population of 176 plants obtained by selfing *R/r* heterozygous plants. We sowed coloured (*R/R* plus *R/r*) and colourless (*r/r*) seeds and in the coloured class we checked for anther colour to distinguish the *R/R* and *R/r* classes, and by so doing we genotyped the population for the *r1* locus. We used SSR molecular markers to complete the mapping: a piece of leaf of each plant was used for DNA extraction (Dellaporta et al. 1983). We used SSR molecular markers chosen on the chromosome 10 (bin 1.05/10.07) from MaizeGDB (http://www.maizegdb.org/ssr.php). The SSR markers chosen were: *bnlg1250* (5'CCATATATTGCCGTGGAAGG3'/ 5'TTCTTCATGCACACAGTTGC3'), *umc1506* (5'AAAAGAAACATGTTCAGTCGAGCG3'/5'ATAAAGGTTGGCAAAACGTAGCCT3'), *phi323152* (5'TCAGGGAGCTCACCTACTACGG3'/5'CACGACTGCACCGATTAGC3'), *bnlg1028* (5'AGGAAACGAACACAGCAGCT3'/5'TGCATAGACAAAACCGACGT3'), *umc1993* (5'CTTTTCTGCTACTCCTGCCTGC3'/5'CTAGCTGATGGAGGCTGTAGCG3'), *umc1196* (5'CGTGCTACTACTGCTACAAAGCGA3'/5'AGTCGTTCGTGTCTTCCGAAACT3'), *bnlg1450* (5'ACAGCTCTTCTTGGCATCGT3'/ 5'GACTTTGCTGGTCAGCTGGT3'), *umc1569* (5'GCAGCTCCAAGTACAGAGGTGAG3'/5'CACTGCAGACACGTAAAATCCAAG3'), *umc2021* (5'AAACTCAAGCTCGGAATGTACTGC3'/

5'CGATACTGATCTACTTCACGCTGG3'). The *bnlg1250, phi323152, bnlg1028*, *umc1993* and *bnlg1450* SSRs were polymorphic and out of them we choose: *bnlg1250, umc1993* and *bnlg1450* to genotype the mapping population. Polymerase chain reactions (PCR) and gel running conditions were performed as described in the previous section. We scored the mapping population for the two traits plant height and ear height. QTL analysis was performed using MAPMAKER/QTL Version 1.1 (Lander et al. 1987).

Results

Development of synthetic populations

We developed and studied for three years two synthetic populations as described in the Materials and Methods section: they differed only for the selection for the *r1* allele present (Supplementary Material Fig.1). To assess whether the different pigmentation observed in the two populations (obtained by crossing the F_1 *r/r* Dekalb 300 hybrid with inbred lines W22 homozygous for *R*) was indeed caused by a monogenic trait we performed a genetic test to analyse the segregation of the coloured/colourless seeds in F_2 families and in backcrosses. These analyses confirmed that the coloured seed trait was a monogenic dominant trait (Supplementary Material Table 2).

Mapping of r1 gene

We used an SSR molecular marker (*bnlg1028*) tightly associated to *r1* gene (about 1 cM) located on the long arm of chromosome 10 to confirm that the presence of pigmentation was determined by presence of the *r1* gene. Mapping analysis was performed using a segregating F_2 population of 221 seedlings obtained by selfing *R/r* heterozygous plants. We classified the three genotypes *R/R*, *R/r* and *r/r* using the difference in tissue-specific pigment accumulation in the seedling (Supplementary Material Table 1). Recombinant values (between the SSR markers *umc1082* and the trait involved in the pigmentation) converted to map distances

using MAPMAKER 3 (Lander et al. 1987) established a distance of about 1.2 cM, according with the known map position of the *r1* gene. By so doing we confirmed that the plants composing the two synthetic populations we produced displayed distinct anthocyanin tissue-specific accumulation patterns: plant tissues colourless with coloured seed (*R/R*) and plant tissues coloured with colourless seed (*r/r*), as shown in Figure 1.

Populations and families studies

The two populations were studied for three field seasons 2006, 2007 and 2008, in the same agronomic conditions (1200 seeds sown in a plot of 140 square meters) and grown in the same agronomic conditions. The data obtained regarding ear weight, ear length, plant height and ear height showed a better performance of the *r/r* synthetic population compared with the *R/R* population (Table 1). To confirm these results we selfed for four times *R/r* plants, obtaining 16 F_4 families (Supplementary Material Fig. 1). Each plant in each family was genotyped for the 3 possible genotypes present: *R/R*, *R/r* and *r/r* (using Supplementary Material Table 1) and then measured for ear weight, ear length and plant height. We observed in all families a positive effect associated with the presence of the *r* allele, also present in the heterozygous *R/r* (Table 2). Furthermore these differences were also observed at the level of seedling fresh weight when the seedlings were cultivated for ten days after germination (Table 3).

Taking these data together, we conjectured that a positive QTL linked to the *r* allele might be responsible for the difference in the performances observed between *r/r* and *R/R* populations. In fact the donor parent of the *r* allele was an F_1 commercial hybrid while the other parent carrying the *R* allele was the W22 inbred line, used only for scientific purposes.

QTL analysis of the long arm of chromosome ten

With the aim of localizing the putative region involved in the phenomenon observed, we performed a QTL experiment using a segregating F_2 population

consisting of 176 plants obtained by selfing *R/r* plants. We analyzed only the region around the *r1* gene using 3 polymorphic SSR molecular markers chosen from the MaizeGDB database and the same *r* trait, taking advantage of the different tissue pigmentation shown by the three genotypes: *R/R*, *R/r* and *r/r* present in the F_2 mapping population. All the plants were scored for plant height and ear height and all molecular and phenotypic data were processed using MAPMAKER/QTL program. The result obtained showed that a QTL associated to these correlated traits (we obtained a LOD of 3.47 for plant height with a variance explained of 11.9 %) seems to map closely linked to the *r1* gene (Fig. 2), furthermore this QTL acts in a dominant way.

Testing a direct R effect

Accordingly, with all the data collected we proposed an alternative hypothesis about the origin of the differences observed among the genotypes studied: we conjectured a direct negative effect of the *R/R* genotype on plant parameters measured, based on the inability of this genotype to synthesized anthocyanins in all plant tissues (with the exception of the seed aleurone layer) as a consequence of the peculiarity of the *R* allele used in this work (*R-sc*). With the aim of testing this hypothesis we studied a different genetic material obtained by crossing the colourless *r/r W64A* inbred line (used only for scientific purposes) with the same coloured inbred lines *W22* homozygous for *R* used previously. We thought that if there was a negative effect due to the absence of anthocyanin in the plant tissues we would observe even in this case (where the *r* allele is not associated to a positive QTL) a difference between the coloured and colourless seedling fresh weight. As shown in Supplementary Material Table 3, we did not observe any difference between coloured and colourless seedlings. To strengthen this result we studied also the progeny obtained by crossing *R-g/r Sn* with Δ/Δ plants (both in W22 genetic background). This cross produced two types of individuals, one bearing the *R-g*/Δ genotype (seed coloured and seedling colourless) and the other bearing the *r Sn*/Δ genotype (seed colourless and seedling highly coloured) and

also in this case no statistical differences were observed between the fresh weights of these two classes of seedlings (Supplementary Material Table 3).

Discussion

The r1 gene encodes for a protein with homology with the basic helix-loop-helix domain (involved in protein dimerization and DNA binding) of the Myc class of oncoproteins regulating the accumulation of anthocyanin in maize tissues (Ludwig et al. 1989; Radicella et al. 1991). This gene is a complex locus composed of two distinct components that independently confer the tissue specific pigmentation patterns of seeds and plants (Robbins et al. 1991). When an allele at the r1 locus carries both the components it is named R-r: R (S per seed component) conferring colored seed and r (P per plant component) conferring coloration in several plant tissues such as root, silk and anther (Supplementary Material Table 1). Practically all commercial maize is colorless because of the presence of the r-r allele, where the S component is inactive whilst the P component is active in conferring pigmentation in different plant tissues.

In this work we crossed the W22 inbred line carrying the R-sc allele (for simplicity named R), conferring coloured seed and colourless plant tissues, with the commercial Dekalb 300 hybrid, carrying the allele r-r (for simplicity named r), the F1 obtained was selfed to produce two synthetic populations differing only for the selection of seed colour as shown in Supplementary Material Fig. 1. We demonstrated by genetic testing that the coloured seed trait was due to the segregation of a single gene acting as a dominant trait (Supplementary Material Table 2) and that this trait was likely the R gene because of the strong association (about 1 cM) with bnlg1028, an SSR molecular marker. These data were in agreement with the observation that the majority of the maize cultivars are homozygous dominant for all the genes of the anthocyanin biosynthetic pathway but carrying weak recessive regulatory alleles (b1, pl1 and r1 alleles) of this pathway the plant tissues accumulate low levels of pigment and the seed is colourless. Thus by crossing these cultivars with lines carrying strong regulatory alleles the F1 obtained is able to accumulate pigment and ¾ of the F2 progeny is

coloured too. Studying these synthetic populations during three seasons we found several differences in agronomic traits such as ear weight, ear length, plant height and ear height (Table 1). The results obtained were confirmed studying 16 single F4 families, and in this case in fact the plants carrying a single dose of r allele (r/r and R/r) showed better performances compared to the R/R plants (Table 2) and this difference was also observed at the level of seedling fresh weight (Table 3). These data were in agreement with the work of Nguetta and Cross (1997) who studied some agronomic traits in maize synthetic populations selected for R-nj, another allele at the r1 locus. In fact they found that by selecting for this seed colour marker there was a correlated positive change in plant and ear measurements such as ear length, ear width, plant height, leaf number and leaf area, suggesting an association between these relevant agronomic traits and the R allele (Cross 1981; Nguetta and Cross 1997). With the aim of investigating the nature of this phenomenon we carried out a QTL experiment, focusing our attention on the long arm of chromosome ten, and using three SSRs and the r1 gene as markers. The results obtained analyzing an R/r F2 segregant population showed that the map position of the QTL associated with plant height and ear height is located close to the position of the r1 gene (Fig. 2). According to the QTL map position we conjectured a direct involvement of the r1 gene, in fact plants homozygous for the R allele are unable to synthesized anthocyanins in all the tissues except in the aleurone layer of the seed (Supplementary Material Table 1 and Supplementary Material Fig. 1) and this lack of pigments could be responsible for the differences observed in the agronomic traits. The anthocyanins and in general the flavonoids, are involved in essential functions in plant biology, acting as phytoalexins in some legumes (Dixon et al. 2001), attracting pollinators (Grotewold et al. 2006), defending from UV-B radiation (Falcone Ferreyra et al. 2007) and conditioning male fertility (Mo et al. 1992). It is also well demonstrated that flavonoids act as regulators of cellular auxin efflux involved in several physiological phenomena (Jacobs and Rubery 1988; Murphy et al. 2002; Taylor and Grotewold 2005; Besseau et al. 2007). A study suggesting a correlation between the genes involved in the flavonoid pathway and yield was carried out by

Frascaroli and Landi who demonstrated that recurrent selection for grain yield changed the allelic frequencies of the P1 gene (regulatory gene involved in the biosynthesis of phlobaphenes, located on the short arm of chromosome 1) in the improved population. In this population the frequency of the P1-wr allele (conferring red cob) increases during the selection for yield improvement with respect to P1-ww allele (conferring white cob) as expected in the case of association of the P1-wr allele with a genetic region important for grain yield or a direct involvement of the activity of this gene in the phenomenon observed (Frascaroli and Landi 1998). To try to address the hypothesis of a direct involvement of anthocyanin pigment in our system, we produced other genetic material and the characterization of the progeny obtained, crossing another colourless r/r inbred line (not used for commercial purpose) with the coloured line R/R previously used, did not reveal any difference in the seedling fresh weight as well as when the seedlings were able to accumulate high level of pigment by presence of the Sn gene (Supplementary Material Table 3). Taken together, these data suggest that an important QTL, explaining more than 10% of the variance associated with important agronomic traits such as plant height and yield, is tightly linked to the r1 gene. This result confirmed the previous work of Koester and co-workers (Koester et al. 1993) who studying two near isogenic lines developed by introgression of Gaspè Flint (a very short, early maize variety) in B73 and SC76 and in selecting for early flowering and plant height they found the importance of the long arm of chromosome 10. More recently this genomic region was picked out studying 142 recombinant inbred lines (RILs) obtained by crossing B73 with H99 inbred lines (Frascaroli et al. 2007). However these studies did not identify a precise map position, whereas in this work we mapped this QTL as being closely linked to the r1 gene.

In concluding, further work will be necessary to assess whether this QTL could be useful in maize genetic improvement, and using our map position and the maize genome sequence data we will try to identify some candidate genes for this quantitative trait.

Acknowledgments

We wish to thank Dr. Davide Reginelli for his hard work in the field. This study was supported by the EU 6th Framework project 007130 "FLORA" and partially by Regione Lombardia – BIOGESTECA project (15083/RCC).

Figures and Tables

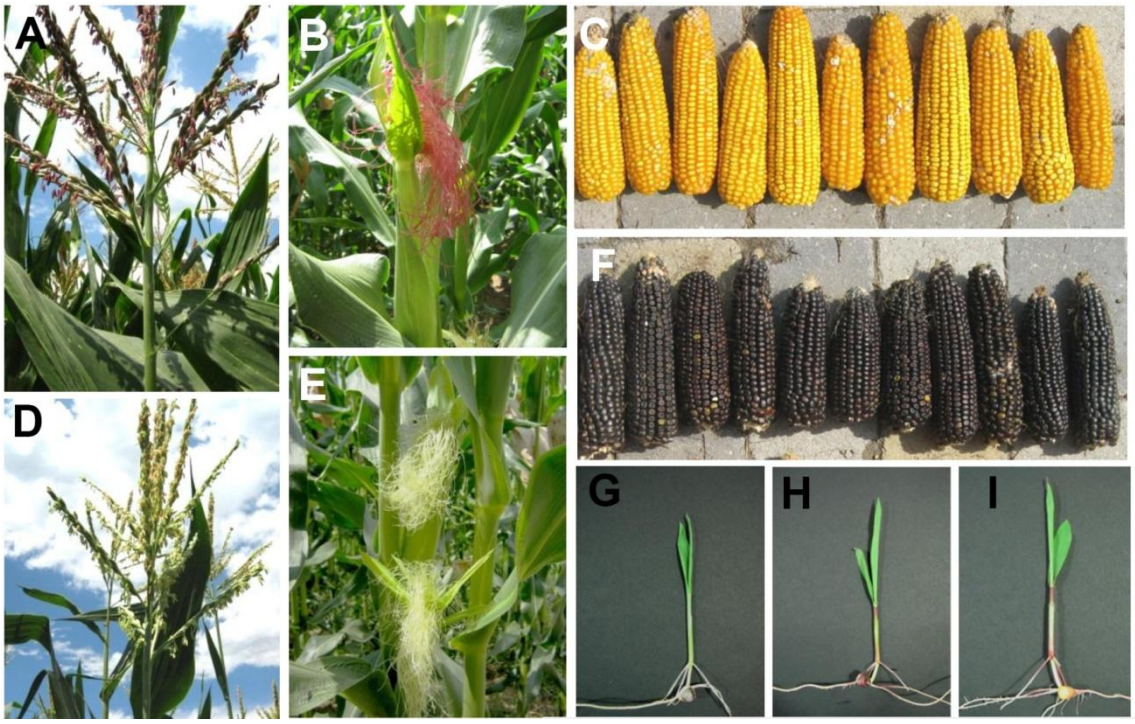


Figure 1: Phenotypic expression and tissue-specificity of *r* and *R* alleles. Phenotype of anther (A), silk (B) and ear (C) in presence of *r* allele and phenotype of anther (D), silk (E) and ear (F) in presence of *R* allele. Seedling phenotype in presence of *R/R* (G), *R/r* (H) and *r/r* (I) genotypes.

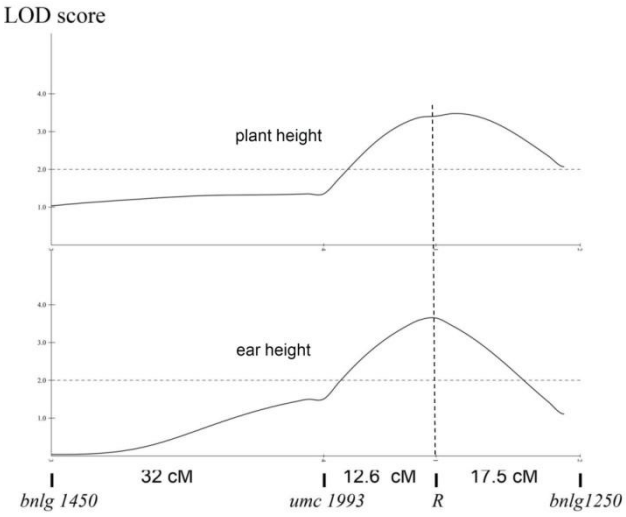
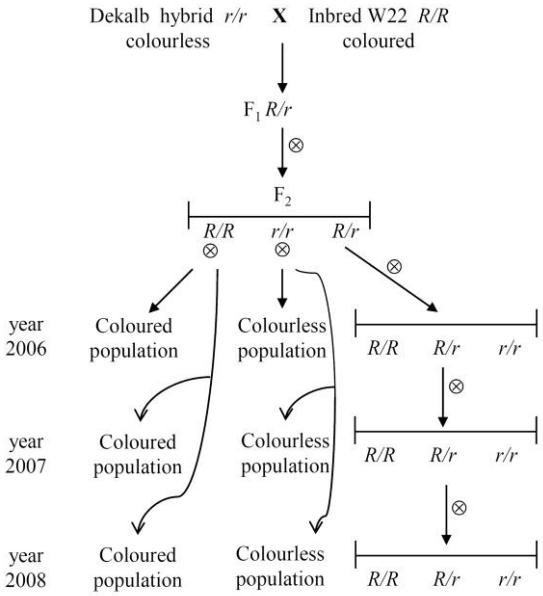


Figure 2. QTL analysis of the long arm of chromosome 10 regarding the plant (above) and the ear (below) height. In abscissa is shown the linkage map obtained using three SSR molecular markers (*bnlg1450*, *umc1993* and *bnlg1250*) and the *R* marker trait. In ordinate is shown the LOD score (logarithm to the base 10 of the likelihood ratio), interval mapping is represented by QTL likelihood plots showing LOD score curve. The horizontal dotted line indicates the LOD threshold of 2.0. Dotted vertical line evidences the *r* gene position. QTL analysis was performed using maximum likelihood methods based on interval mapping using MAPMAKER-QTL program.



Supplementary Material Figure 1.
Pedigree scheme showing the development of *R/R* and *r/r* synthetic populations and *R/r* families. We selfed the F_2 progeny selecting *R/R* (coloured seed, colourless anther), *r/r* (colourless seed, coloured anther) and *R/r* (coloured seed, coloured anther) plants

Table 1. Means of ear weight, ear length, plant height, ear height in coloured (*RR*) and colourless (*rr*) control synthetic populations over 3 years. Confidence intervals at 95% are shown.

year	ear weight (g)		ear length (cm)		plant height[1] (cm)		ear height (cm)	
	R/R	*r/r*	*R/R*	*r/r*	*R/R*	*r/r*	*R/R*	*r/r*
2006	61.39 ± 2.48	77.84 ± 3.29*	12.05 ± 0.23	13.61 ± 0.24*	n.t.[2]	n.t.	n.t.	n.t.
2007[3]	97.55 ± 2.42	120.89 ± 4.70*	13.98 ± 0.21	15.18 ± 0.31*	210.25 ± 4.94	229.03 ± 5.32*	112.88 ± 2.53	125.23 ± 3.57*
2008	60.82 ± 5.46	71.13 ± 4.84*	12.02 ± 0.43	13.65 ± 0.37*	156.24 ± 4.40	177.48 ± 5.20*	75.42 ± 2.15	90.66 ± 3.21*

[1] plants height was recorded at the level of the flag leaf ; [2]n.t. not tested; [3]with nitrogen fertilization (240 kg/ha)

* confidence interval significantly different from *R/R* at $P < 0.05$, $n>700$

Table 2. Measurement of ear weight, ear length and plant height of mature plants having the genotypes *R/R*, *R/r* and *r/r*, obtained selfing *R/r* individuals (16 families). Confidence intervals at 95% are shown.

genotype	ear weight (g)	ear length (cm)	plant height (cm)[1]
R/R	72.32 ± 4.16	12.23 ± 0.38	168.84 ± 4.27
R/r	81.16 ± 3.04*	12.85 ± 0.25	177.67 ± 3.97*
r/r	81.98 ± 3.34*	12.98 ± 0.27*	180.87 ± 2.66*

[1] plants height was recorded at the level of the flag leaf

*confidence interval significantly different from *R/R* at $P < 0.05$, $n>100$

Table 3. Measurement of fresh weight of *R/R*, *R/r* and *r/r* seedlings obtained selfing *R/r* plants. Measurements were made after ten days of germination at 25°C. Confidence intervals at 95% are shown.

genotype	*R/R*	*R/r*	*r/r*
seedling fresh weight (g)	1.05 ± 0.10	1.34 ± 0.08*	1.30 ± 0.11*

*confidence interval significantly different from *R/R* at $P < 0.05$, $n>100$

Supplementary Material Table 1. Tissue-specific expression of the three genotypes *R/R*, *R/r* and *r/r*.

genotype	anthocyanin pigmentation				
	seed	root	culm	anther	silk
R/R	+[1]	-[2]	-	-	-
R/r	+	+	+/-[3]	+	+
r/r	-	+	+/-	+	+

[1]pigmented tissue; [2]colourless tissue; [3]weakly pigmented tissue

Supplementary Material Table 2. Segregation of the coloured/colourless seeds observed in genetic tests. The data showed that the coloured seed trait is a monogenic dominant trait.

cross	segregation			
	coloured	colourless	χ^2	P
R/r ⊗	3859	1296	0.054	0.90-0.70
R/r X *r/r*	2737	2742	0.004	0.95-0.90

Supplementary Material Table 3. Measurement of seedling fresh weight in the progenies obtained selfing *R/r* plants (W64A genetic background) and crossing *R-g/rSn* with Δ/Δ plants (both in W22 genetic background). Measurements were made after ten days of germination at 25° C. Confidence intervals at 95 % are shown.

cross	genotype in the progeny	seedling anthocyanin pigmentation	seedling fresh[1] weight (g)
R/r ⊗	*R/R*	-[2]	1.01 ± 0.05
	R/r	+[3]	1.07 ± 0.02
	r/r	+	n.t.[4]
R-g/r Sn X Δ/Δ	*R-g*/Δ	-	0.97 ± 0.09
	r Sn/Δ	+[5]	0.87 ± 0.08

[1]in the first cross for each genotype n>50, whilst in the second cross n>20; [2]colourless tissue; [3]pigmented tissue; [4] n.t. not tested
[5] strongly pigmented tissues

References

Alleman M, Kermicle JL (1993) Somatic variegation and germinal mutability reflect the position of transposable element dissociation within the maize *R* gene. Genetics 135:189-203

Beavis WD, Grant D, Albertsen M et al. (1991) Quantitative trait loci for plant height in four maize populations and their associations with qualitative genetic loci. Theor Appl Genet 83:141-145

Besseau S, Hoffmann L, Geoffroy P et al. (2007) Flavonoid accumulation in arabidopsis repressed in lignin synthesis affects auxin transport and plant growth. Plant Cell 19:148-162

Bouchez A, Hospital F, Causse M et al. (2002) Marker-Assisted Introgression of favorable alleles at Quantitative Trait Loci between maize elite lines. Genetics 162:1945-1959

Campos H, Cooper M, Habben JE et al. (2004) Improving drought tolerance in maize: a view from industry. Field Crops Research 90:19-34

Chandler VL, Radicella JP, Robbins Chen JC et al. (1989) Two regulatory genes of the maize anthocyanin pathway are homologous: isolation of *B* utilizing *R* genomic sequences. Plant Cell 1:1175-1183

Chardon F, Virlon B, Moreau L et al. (2004) Genetic architecture of flowering time in maize as inferred from quantitative trait loci meta-analysis
and synteny conservation with the rice genome. Genetics 168:2169-2185

Consonni G, Ronchi A, Pilu R et al. (1997) Ectopic anthocyanin pigmentation in maize: a tool for defining interactions between homologous regulatory factors. Mol Gen Genet 225:401-410

Cross HZ (1981) Use of *R-nj* aleurone color to improve grain yields of early maize. Crop Sci 21:751-754

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19-21

de Vlaming P, Schram AW, Wiering H (1983) Genes affecting flower colour and pH of flower limb homogenates in Petunia hybrida. Theor Appl Genet 66:271-278

Dixon RA (2001) Natural products and plant disease resistance. Nature 411:843-847

Dooner HK, Kermicle JL (1974) Reconstitution of the *R-r* compound allele in maize. Genetics 78:691-701

Dooner HK, Robbins TP, Jorgensen RA (1991) Genetic and developmental control of anthocyanin biosynthesis. Annu Rev Genet 25:173-199

Falcone Ferreyra ML, Rius S, Emiliani J et al. (2007) Cloning and characterization of a UV-B-inducible maize flavonol synthase. Plant Cell 19:148–162

Frascaroli E, Cane` MA, Landi P et al. (2007) Classical genetic and quantitative trait loci analyses of heterosis in a maize hybrid between two elite inbred lines. Genetics 176:625-644

Frascaroli E, Landi P (1998) Allelic frequency change of *P1* gene in a maize population after recurrent selection for grain yield. Crop Sci 38:1391-1394

Grotewold E. (2006) The genetics and biochemistry of floral pigments. Annu Rev Plant Biol 57:761–780

Jacobs M, Rubery PH (1988) Naturally occurring auxin transport regulators. Science 241:346-349

Kermicle JL (1984) Recombination between components of a mutable gene system in maize. Genetics 107:489-500

Koester RK, Sisco PH, Stuber CW (1993) Identification of quantitative trait loci controlling days to flowering and plant height in two isogenic lines of maize. Crop Sci 33:1209-1216

Lander ES, Green P, Abrahamson P et al. (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181

Lawrence CJ, Harper LC, Schaeffer ML, Sen TZ et al. (2008) Maize GDB: the maize model organism database for basic, translational, and applied research. Int J Plant Genomics DOI:10.1155/2008/496957

Long Y, Zhang C, Meng J (2008) Challenges for QTL analysis in crops. Crop Sci Biotech 11:7-12

Ludwig SR, Habera LF, Dellaporta SL et al. (1989) *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production encodes a protein similar to transcriptional activators and contains the myc-homology region. Proc Natl Acad Sci USA 86:7092-7096

Mather K (1941) Variation and selection of polygenic characters. J Genet 41:159-193

Mo Y, Nagel C, Taylor LP (1992) Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen.
Proc Natl Acad Sci USA 89:7213–7217

Multani DS, Briggs SP, Chamberlin MA et al. (2003) Loss of an MDR transporter in compact stalks of maize *br2* and sorghum *dw3* mutants. Science 302:81-84

Murphy AS, Hoogner KR, Peer WA et al. (2002) Identification, purification, and molecular cloning of N-1-naphthylphthalmic acid-binding plasma membrane-associated aminopeptidases from Arabidopsis. Plant Physiol 128:935-950

Nguetta ASP, Cross HZ (1997) Correlated responses in ear and plant traits in maize synthetics selected for *R-nj* color expression. Crop Sci 37:739-744

Paterson A, Lander ES, Hewitt JD et al. (1988) Resolution of quantitative traits into mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. Nature 335:721-726

Peng J, Richards DE, Hartley NM et al (1999) 'Green revolution' genes encode mutant gibberellin response modulators. Nature 400:256-261

Pilu R, Piazza P, Petroni K et al. (2003) *pl-bol3*, a complex allele of the anthocyanin regulatory *pl1* locus that arose in a naturally occurring maize population. Plant J 36:510-521

Pilu R, Cassani E, Villa D et al. (2007) Isolation and characterization of a new mutant allele of *brachytic 2* maize gene. Mol Breeding 20:83-91

Radicella JP, Turks D, Chandler VL (1991) Cloning and nucleotide sequence of a cDNA encoding *B-Peru*, a regulatory protein of anthocyanin pathway in maize. Plant Mol Biol 17:127-130

Robertson DS (1985) A possible technique for isolating genomic DNA for quantitative traits in plants. J Theor Biol 117:1-10

Robbins TM, Walker EL, Kermicle JL et al. (1991) Meiotic instability of the *R-r* complex arising from displaced intragenic exchange and intrachromosomal rearrangement. Genetics 129:271-283

Roth BA, Go SA, Klein TM et al. (1991) *C1*- and *R*-dependent expression of the maize *Bz1* gene requires sequences with homology to mammalian myb and myc binding sites. Plant Cell 3:317-325

Stuber CW, Edwards MD, Wendel JF (1987) Molecular marker-facilitated investigations of quantitative trait loci in maize. II Factors influencing yield and its component traits. Crop Sci 27:639-648

Szalma SJ, Hostert BM, LeDeaux JR et al. (2007) QTL mapping with near-isogenic lines in maize. Theor Appl Genet 114:1211-1228

Taylor LP, Grotewold E (2005). Flavonoids as developmental regulators. Curr Opin Plant Biol 8:317-323

Tuberosa R, Salvi S (2006) Genomics-based approaches to improve drought tolerance of crops. Trends Plant Sci 11:405-412

Winkel-Shirley B (2002) Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol 5:218-223

Study of maize genotypes rich in anthocyanins for human and animal nutrition

Chiara Lago[1], Elena Cassani[1], Roberto Pilu[1].

[1] Dipartimento di Produzione Vegetale, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy.

This is a pre-copy-editing, author-produced of an article accepted for publication in MINERVA BIOTHECNOLOGICA *following peer review.*

Lago C, Cassani E, Pilu R (2011). Study of maize genotypes rich in anthocyanins for human and animal nutrition. *Minerva Biotec* **23** (2 Suppl. 1):34-6.

The definitive publisher-authenticated version is available online at:
http://www.minervamedica.it/en/journals/minerva-biotecnologica/article.php?cod=R04Y2011N05A0034

Introduction

Flavonoids, a group of secondary metabolites belonging to the class of phenylpropanoids, play essential functions in plant. There is evidence that flavonoids may function in plants to screen harmful radiations, bind phytotoxins, and help to regulate the stress response by controlling auxin transport[1]. Besides, in the last years, the interest in the phytochemical constituents has increased due to consumer awareness of their health benefits. In particular dietary flavonoids have received considerable attention since epidemiological studies suggested that regular consumption of flavonoid-rich foods or beverages is associated with a decreased risk of cardiovascular mortality. Among the flavonoids, it seems that anthocyanins might function as potent in vivo antioxidants: their long-term dietary potential health benefits can be proved by the use of plants that accumulate specific anthocyanin[2]. Maize could be an example of these functional foods: purple and blue corn are pigmented varieties rich in anthocyanin originally cultivated in South America. In maize, anthocyanin are synthesized by a complex pathway made up of more than 20 genes and regulated by two classes of transcription factors, that is *R1/B1* bHLH genes and *C1/Pl1/P1* MYB gene families[3]. The presence of dominant alleles of these regulatory genes is necessary to accumulate the pigments. Due to their important benefits on human and animal health, the aim of this project is to better understand the regulatory mechanisms of the anthocyanin pathway in order to obtain corn lines with the highest amount of these pigments.

Materials and Methods

Plant Samples

The analysis here reported were performed on kernels from ears of the two NILs: R2723 and R2724. These two NILs derived from 6 cicles of self-pollination, after a cross between the B73 inbred line and the W23 line carrying B/Pl regulatory genes of anthocyanin pathway. The two NILs were sown in adjacent rows in 2010, under

the same agronomic conditions, in the experimental field of the University of Milan located in Landriano (PV).

Germination test

The maize ears of both lines were sampled with a progressive number (from 1 to 11 and from 21 to 23 for R2723 and from 1 to 13 for R2724). We measure the mean weigh of 15 seeds coming from each ear. Ten kernels for everyone were weighted and germinated in darkness for 4 days at 24°C to check the germination percentage.

Anthocyanins quantification

From each ear 4 kernels were taken, cleaned from the glumes and grinded down to a fine powder with a ball mill. About 5 mg of the powder was boiled with 100 µl of distilled water for 30 minutes. Then we added 1ml of the extraction buffer (1% HCl in 95% ethanol) and the sample were left in over night agitation. After a centrifugation at 13000rpm for 10 minutes, the surnatants were collected and the pellets were put again in agitation for 2 hours in 500 µl of exctraction buffer. Finally we collected the surnatants together and after a centrifugation at 13000rpm for 30 minutes, their absorbance was determined spectrophotometrically at λ= 530 nm. The amount of anthocyanin was calculated as cyanidin 3-glucoside equivalents (molar extinction coefficient (e) 26900 L m-1 mol-1, MW 449.2) for g of dry flour.

Pericarp measurements

Seeds of the ears that showed the highest and the lowest anthocyanin amount of each line were cut in a half. The pericarp thickness was measured using a stereoscope equipped with a CCDcamera.

Results

In order to improve the anthocyanin level in the maize kernels, we obtained two NILs (named: R2723 and R2724) after 6 cicles of self-pollination, starting from a cross between an inbred line and a line carrying the regulatory genes for anthocyanin accumulation in the pericarp (B and Pl). We sampled 15 ears from the line R2723 (numbered from 1 to 12 and from 21 to 23) and 13 ears from the line R2724 (numbered from 1 to 13). The 2 NILs produced ears showing a very high level of pigmentation in the kernel, in fact we have extracted and quantified the anthocyanin content from each ear of both lines: in the ears of the R2723 line the pigment content ranged from 0,749 mg cyanidin/g (ear 23) to 1,78 mg cyanidin/g (ear 7) while the cyanidin content of the NIL R2724 ranged from 0,479 mg cyanidin/g (ear 7) to 2,89 mg cyanidin/g (ear 10). The ears of the R2724 line showed a high variation in anthocyanin levels compared to the R2723 line (Fig. I). With the aim to understand the nature of this large variation in the anthocyanin content in these NILs we measured in each ears the seed weight, the germination rate and the pericarp thickness.

The data we collected showed no correlation between the anthocyanin content and the seed weight and the germination rate (Fig. II). For what it concerns the pericarp thickness, we measured 4 half-seeds for each of the following ears: R2723(23), R2723(7), R2724(7), R2724(10). The values measured ranged from 31,99 μm to 50,62 μm. As shown in Fig. III, the ear with the higest anthocyanin level had also the highest pericarp thickness, nevertheless this very low variation in pericarp thickness can not explain the large variation in anthocyanin content.

Discussion

With the aim to improve our knowledge about the maize ability to accumulate anthocyanin (so that to obtain corn lines with a very high amount of these pigments) we have analysed two NILs homozygous for *B* and *Pl* genes. After 6 cycles of self-pollination the differences in the genetic background in each line are expected to be very low, nevertheless we noted a dramatic variation in the anthocyanin content among different ears of the line R2724 (Fig. I); the hypothesis that the differences on anthocyanin levels could be explained by differences in the

seeds weight or in the pericarp thickness was refuted on the basis of the collected data (Fig II and III). Hence we hypothesized an involvement of an epigenetic phenomenon to explain this variation. It is known in fact that the regualtory genes of the anthocyanin biosynthetic pathway are susceptible of silencing processes[4], thus the variation in R2724 line could be due to a partial switch off of the anthocyanin pathway regulatory genes. Further analysis will be necessary to confirm this hypothesis (in particular expression analysis of regulatory genes) and for better understanding of this phenomenon.

Acknowledgements

We wish to thank Dr. Davide Reginelli, Dr. Francesco Cerino Badone and Dr. Andrea Bucci for their hard work in the field.

Figures and Tables

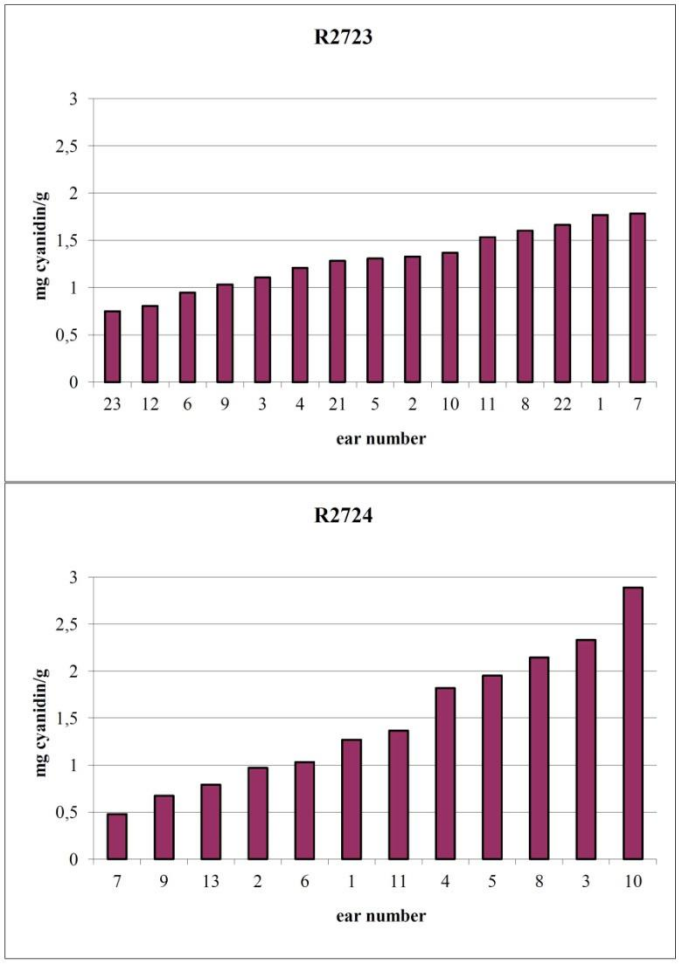


Figure I. Anthocyanin levels of each ear in the R2723 (above) and R2724 (below) lines.

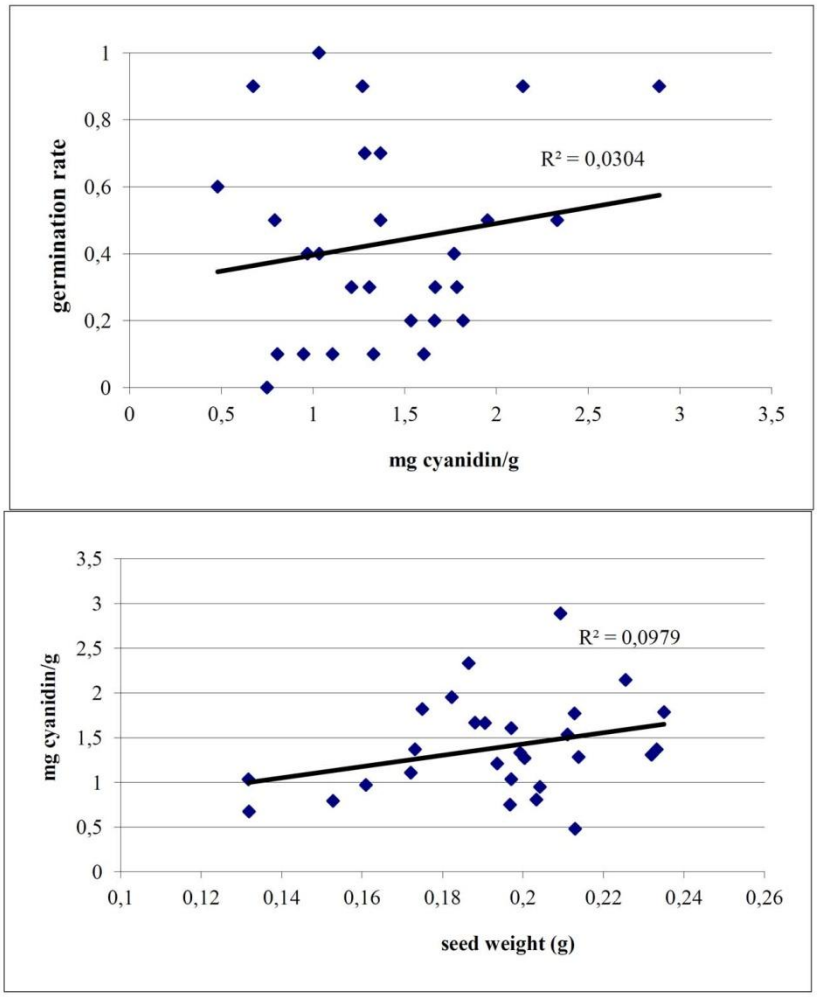


Figure II. Correlation analysis between the anthocyanin content with the germination capability (above), and the mean of seed weight with the antocyanin content (below).

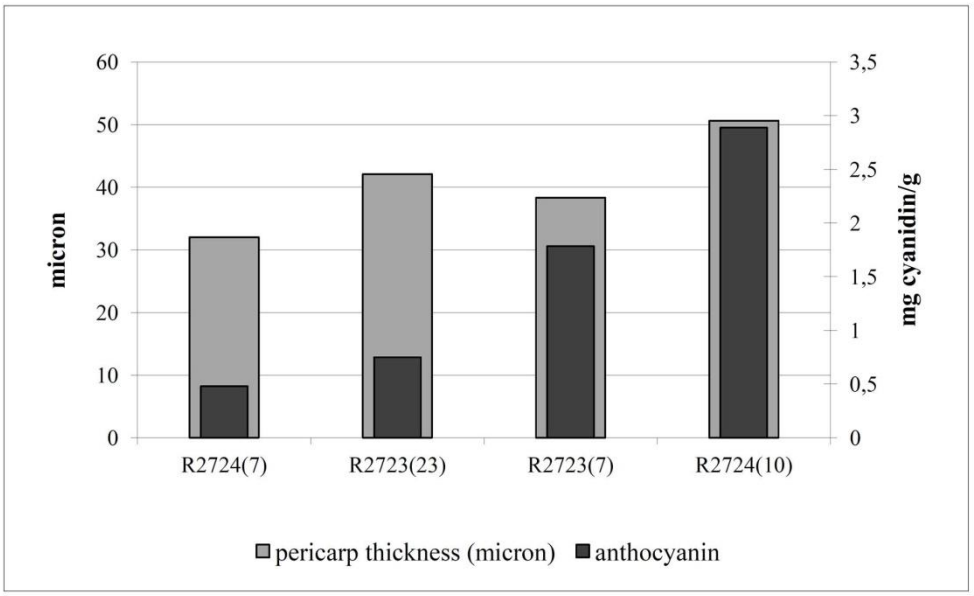


Figure III. Variation in the pericarp thickness (on the left) compared to the variation of the ears with the highest and the lowest anthocyanin levels (on the right) of both lines.

References

1. Winkel-Shirley B. Biosynthesis of flavonoids and effects of stress. Current opinion in plant biology 2002; 5:218-223.

2. Toufektsian MC, de Lorgeril M, Nagy N, Salen P, Donati MB, Giordano L, Mock HP, Peterek S, Matros A, Petroni K, Pilu R, Rotilio D, Tonelli C, de Leiris J, Boucher F, Martin C. Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. J. Nutr. 2008;138:747–752.

3. Cerino Badone F, Cassani E, Landoni M, Doria E, Panzeri D, Lago C, Mesiti F, Nielsen E, Pilu R. The *low phytic acid1-241* (*lpa1-241*) maize mutation alters the accumulation of anthocyanin pigment in the kernel. Planta 2010;231:1189–1199.

4. Hollik JB, Patterson GI, Coe EHJr, Cone CK, Chandler VL. Allelic interaction heritably alter the activity of a metastable maize pl allele. Genetics 1995;141:709-719.

Study and characterization of a novel functional food: purple popcorn

Chiara Lago[1], Michela Landoni[2], Elena Cassani[1], Enrico Doria[3], Erik Nielsen[3], Roberto Pilu[1].

[1] Dipartimento di Scienze Agrarie e Ambientali - Produzione, Territorio, Agroenergia, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy
[2] Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy
[3] Dipartimento di Genetica e Microbiologia, Università degli Studi di Pavia, Via Ferrata 1, I-27100 Pavia, Italy

This is a pre-copy-editing, author-produced of an article accepted for publication in MOLECULAR BREEDING *following peer review.*

Lago C, Landoni M, Cassani E, Doria E, Nielsen E, Pilu R. Study and characterization of a novel functional food: purple popcorn (2013). Mol Breeding **31**:575–585.

The definitive publisher-authenticated version is available online at:
http://link.springer.com/article/10.1007/s11032-012-9816-6

Abstract

Many phytonutrients seem to be able to fight the oxidant effect which may lead to chronic diseases. Among them anthocyanins have been studied for a long time, and different type of functional food rich in these pigments are already available on the market. In particular wine, berries and various cereals have already aroused consumers' awareness, and in this context we propose a new and attractive healthy food: purple popcorn. Popcorn is the most popular American snack, now well known all over the world. A corn rich in anthocyanins, suitable to be transformed into a snack could help to introduce healthy antioxidant compounds in the diet of many people, contributing to the prevention of chronic diseases. In this work we developed a coloured pop corn variety enriched in anthocyanins (about 66 mg/100g, mainly cyanidin) by recurrent selection scheme with the aim to obtain an healthier snack. The selection was based on some quality characteristics such as anthocyanins content, popping abilitiy and the PEV (Popping Expansion Volume). The purple pop corn obtained was further analyzed by HPLC (High Pressure Liquid Chromatography) and the DPPH radical scavenging ability, prior and after the microwave treatment. The results obtained showed that even if the microwave treatment reduced the anthocyanins content of about 46%, the remaining anthocyanins exhibited a remarkable antioxidant capacity compared to the colourless control. Finally it was also checked the taste perception by comparing coloured vs uncoloured popcorn and no difference was perceived.

Key words: *Zea mays*, *Purple plant 1*, *Booster 1,* anthocyanins, functional food

Introduction

With the lengthening of the human life span, the mortality from chronic noncommunicable diseases is increasing and much scientific evidence links them to socio-behavioural and dietary habits (Martin et al. 2011; WHO Noncommunicable diseases country profiles 2011 ISBN 978 92 4 150228 3). In fact while the consumption of unhealthy foods can promote this kind of mortality (Martin et al. 2011), several studies have proven that many phytonutrients and other metabolites supplied by food exert a direct antioxidant effect, useful to protect human health by preventing chronic diseases (Virgili and Marino 2008). The world renowned example of the French paradox stated that a moderate consumption of red wine rich in antioxidant molecules such as resveratrol, flavonols, anthocyanins and catechins can decrease the probability of mortality from cardiovascular diseases, even with a dairy rich diet such as the French one (Lippi et al. 2010a, 2010b; Martin et al. 2011). The anthocyanin molecules, the famous red, blue and purple pigments of fruits and flowers, have been deeply studied in wine (de Pascual-Teresa and Sanchez-Ballesta 2008), but not only in that product.

Even corn (Zea mays L.) is able to accumulate anthocyanins, and the most abundant is cyanidin-3-glucoside (Escribano-Bailon et al. 2004; de Pascual-Teresa et al. 2002; Nakatani et al. 1979). More than 20 genes have been discovered to play a structural or regulatory role in this biosynthetic pathway in maize (Chandler et al. 1989; Dooner et al. 1991; Pilu et al. 2003). The reactions catalysing the biosynthesis of anthocyanins take place in the cytosol, then these pigments are stored in the vacuole, probably with the recruitment of the protein encoded by ZmMRP3 gene (Goodman et al. 2004). The expression of the structural genes is regulated by two multigene families the r1/b1 belonging to the class of bHLH transcription factors and the c1/pl1/p1 belonging to the class of MYB transcription factors (Chandler et al. 1989; Dooner et al. 1991; Pilu et al. 2003; Pilu et al. 2012). A member of each family must be present and active in the dominant form to activate anthocyanin biosynthesis and according to the combination of alleles of the regulatory genes, the synthesis will be active in different plant

tissues. The typical purple and blue colours of tropical maize seeds are due to the anthocyanin regulatory genes B/Pl that confer the colour to the pericarp and also to the plant (Chandler et al.1989; Bodeau and Walbot 1992; Gaut 2001; Pilu et al. 2003; Pilu 2011).

Purple maize is historically cultivated in South America, principally in Peru and Bolivia, and used for the traditional drink "Chicha Morada" (Schwarz et al. 2003) but also for making purple tortillas chips and as a food colorant (Escribano-Bailon et al. 2004).

Another ancient use of maize was as red, blue and yellow popcorn (Grobman et al. 2012): it seems that the Native American tribes ate it and also used it as a clothing accessory (in 'Popcorn Profile' , Hansen R., content specialist, AgMRC, Iowa State University). So popcorn is the original American snack and the yellow one has become one of the favourite snack foods not only in U.S.A, but also in a large part of the world (Karababa 2006).

In U.S.A. it became popular during the Great Depression, while from 1970 to 1990 popcorn sales increased by 2 to 8 %, with a quickening of the domestic demand in the 1980s thanks to the introduction of microwave popcorn which by 1999 represented 72% of all popcorn sales (http://www.agmrc.org/commodities__products/grains__oilseeds/corn_grain/popcorn_profile.cfm). Among the snacks, popcorn is one with the best nutritional features: according to the USDA-NAL tables it provides 78.77% of carbohydrates, 12.94% of proteins, 4.54% of fats with also high amounts of iron and calcium (3.19 mg and 7 mg respectively per 100 g) (http://ndb.nal.usda.gov/ndb/foods). Ziegler et al. (1984) underlined the fact that without dressing popcorn has low calories, gives benefits to the teeth and its hull as bran contributes 14.5g per 100g.

However in recent years consumption decreased compared to that of other snack foods, but Americans still continue to consume about 52 quarts of popped popcorn per capita per year (http://www.agmrc.org/commodities__products/grains__oilseeds/corn_grain/popcorn_profile.cfm).

Considering popcorn's high popularity on one side and the antioxidant ability of the anthocyanins that maize could accumulate on the other, we decided to develop coloured popcorn. The aim was to obtain popcorn coloured lines and consequently hybrids with high yield, high anthocyanin content and capable of producing popped corn of excellent quality. A measure for the quality is the expansion volume (Song et al. 1991; Soylu and Tekkanat 2007). 'Popping expansion' refers to the unique ability of popcorn, among all the types of maize, to explode after heating, forming large edible flakes (Ziegler 2001); it is defined as the ratio between the popped corn volume and the original unpopped one (Robbins and Ashman 1984).

Although yield is an important trait, it is the popping expansion property that has to excel: commercial popcorn is sold by volume, but it is bought by weight (Ceylan and Karababa 2002).

In this work we present the results of a study in which a new popcorn coloured line is compared with the traditional yellow one, while considering some characteristics which play important roles in the definition of popcorn quality. We also compared the anthocyanins content and antioxidant activity of the coloured popcorn line before and after the microwave treatment and we report on consumer acceptance of the new popcorns.

Materials and Methods

Plant material

To develop a new coloured popcorn maize, we crossed a typical commercial yellow popcorn line with a source of the tropical anthocyanin biosynthesis regulatory genes *Booster1* (*B)* and *Purple plant1* (*Pl)* in the homozygous state, which determine pericarp and plant colour. Five cycles of backcrossing with the recurrent yellow parent and 3 cycles of self pollination were performed in the experimental field of the University of Milan located in Landriano (PV, Italy). The

selection of heterozygous plants in the backcrosses were guided by the anthocyanin content and by Molecular Assisted Selection (MAS).

Molecular Marker assay

DNA was extracted from the leaves of parental (P1 and P2) and progenies' plants, as previously described (Dellaporta et al. 1983). The *nc009* SSR molecular marker (5'CGAAAGTCGATCGAGAGACC3'/ 5'CCTCTCTTCACCCCTTCCTT3') part of the *pl1* gene located on chromosome 6 and the *umc1776* SSR molecular marker (5'AAGGCTCGTGGCATACCTGTAGT3'/5'GCTGTACGTACGGGTGCAATG3') part of the *b1* gene on the short arm of chromosome 2 from MaizeGDB (http://www.maizegdb.org/ssr.php) were used. The *umc2528* SSR (5'CTCATCAACATGCAAAGGACGTAG3'/5'ATTCAAATGCCTCTAAGCTAGCCG3') was also used: it is associated to the *r* gene on the chromosome 10 not directly involved in the selection. Polymerase Chain Reactions (PCR) and gel running conditions were performed as described in the SSR Methods Manual by MaizeGDB
(http://www.maizegdb.org/documentation/maizemap/ssr_protocols.php).

Anthocyanin quantification

Anthocyanin quantification was made using 4 kernels for each line to be analysed, they were cleaned from the glumes and ground down to a fine powder with a ball mill. Five mg of the powder was boiled with 100 µl of distilled water for 30 minutes. Then we added 1 ml of the extraction buffer (1% HCl, 95% ethanol) and the samples were left overnight in agitation. After a centrifugation at 13000rpm for 10 minutes, the supernatants were collected and the pellets were again put in agitation for 2 hours in 500 µl of extraction buffer. Finally we collected the supernatants together and after a centrifugation at 13000rpm for 30 minutes, their absorbance was determined spectrophotometrically at □□□530 nm.
The amount of anthocyanin was calculated as cyanidin-3-glucoside equivalents (molar extinction coefficient (e) 26900 L m^{-1} mol^{-1}, MW 449.2) for 100 g of dry flour.

The same procedure was carried out on the seeds and on the corresponding popped flakes of the best anthocyanin accumulator popcorn line and replicated three times. The confidence interval (C.I.) at 95% was calculated.

Germination test

We calculated the mean weight by weighing 30 seeds from the best anthocyanin accumulator popcorn line and 30 seeds from a yellow popcorn line, as a control. These seeds were then germinated in Plexiglas boxes on filter paper imbibed with water, in darkness for 4 days at 24°C to check the germination percentage. Confidence intervals at 95% were calculated.

Popping test

To test the popping ability 99 seeds from the best anthocyanin accumulator line and from yellow control line were put in a paper bag inside a home microwave. 800 watt were imposed for 2 minutes and 10 seconds, then the popping percentage was counted and the confidence intervals at 95% were calculated.

Determination of flake and kernel volume and estimation of the Popping Expansion Volume (PEV)

The volume of ten kernels of both coloured and colourless popcorn lines was measured with a liquid pycnometer working with deionised water. The measure was repeated 5 times. After the microwave treatment, ten flakes of the popped kernels of the coloured and the colourless maize were put in a graduated cylinder half filled with ethanol. The flake volume was measured as the difference in the ethanol level before and after the popcorns were added. The measure was repeated 6 times and statistical analysis was performed to calculate the mean values of the flakes' volume and the corresponding confidence intervals at 95%.
PEV, defined as the ratio of popped corn volume to original unpopped corn volume, according to Robbins and Ashman (1984) and Babu et al. (2006) was calculated.

Extraction and qualitative determination of anthocyanins

TLC

The coloured pericarp layer of five kernels of the coloured popcorn line were ground down to a fine powder; the same procedure was performed using five coloured flakes, after the microwave treatment. They were boiled at 100°C with 2 ml of 2N HCl for 40 minutes. After ice freezing, 1 ml of isoamyl alcohol was added. The upper phase was dried and suspended in EtOH 95% and HCl 1%. The extracts and the cyanidin, pelargonidin and delphinidin standards were loaded on a pre-coated plastic sheet (POLYGRAM CEL 300, MACHEREY-NAGEL) for Thin Layer Chromatography (TLC) using formic acid:HCl:water 5:2:3 as solvent. Developed plates were dried and pictures were taken with a digital camera (A430 Canon) using both white and UV illumination.

HPLC

For HPLC analysis we used the same procedure described for TLC extraction, but the dried samples were then suspended in methanol. After the extraction process, 20 µl of the sample were injected in HPLC Kontron Instrument 420 system equipped with a C18 column Zorbax ODS column, 250 mm X 4.6 mm, 5 □m, Teknokroma (Agilent Technologies, Santa Clara, CA, USA) and the absorbance at 530 nm was monitored. Anthocyanin quantification was performed by the method used by Astadi (Astadi et al. 2009); the HPLC conditions were as follows: from min 0 to 8 min, Solvent A (10% formic acid) from 96 to 85%, B (100% Acetonitrile) from 4 to 15%; from min 8 to 25, Solvent B was kept at 15%; from min 25 to 27, A 20%, B 80%; from min 27 to 30, A 80%, B 20%. The flow rate was 1 ml/min.

Antiradical ability

The antioxidant ability of the anthocyanin extracts was determined as the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging activity of each sample (Brand-Williams et al. 1995; Cevallos-Casals and Cisneros-Zevallos 2003; Leong and Shui 2002; Hu et al. 2004; Yang and Zhai, 2010). To an equal amount of pericarp powder of coloured and uncoloured, popped and unpopped samples

was added acetone 70% (acetone:water 70:30 v/v) and the mixture was shaked at 4°C for 3 days. The extracts were centrifuged for 10 minutes at 13000rpm, their anthocyanin concentration was equalized between the two coloured samples and the 2 colourless ones and finally they were conveniently diluted. Increasing aliquots of each sample were added to a 0.12 mM ethanolic DPPH solution to reach the final volume of 2.50 ml. The absorbance of the discolorations of the DPPH in ethanol and of the samples were measured at 516 nm after incubation for 3 hours at room temperature in the dark.

The percentage of DPPH was calculated as: % DPPH = (Ac−As) × 100 / Ac

where Ac is the absorbance of the control, and As is the absorbance of each increasing aliquot of the sample. (Leong and Shui, 2002; Hu et al. 2004; Yang and Zhai 2010). To check whether microwave treatment modified anthocyanin content, a curve was traced by interpolating the increasing aliquots of an extract with the corresponding DPPH scavenged percentage.

Panel Test/consumer test

Sixty-seven subjects were randomly recruited for a consumer test. The new developed coloured popcorns underwent a microwave heating for 2 minutes and 10 seconds to obtain the popped flakes; the same treatment was performed for the commercial yellow popcorns. No salt, nor other dressings were added to the flakes. The blinded subjects tasted two flakes for each kind of popcorn and expressed their judgment according to a scale from 1, the worst, to 10, the best. The mean, the median and the mode of the judgments relative to the two different kind of popcorn were calculated.

Results

Development of colored popcorn lines through Molecular Assisted Selection (MAS)

With the aim of generating new popcorn lines we adopted a recurrent selection scheme with the help of Molecular Assisted Selection (MAS). We used a black line

of maize as a source of the tropical *B* and *Pl* anthocyanin regulatory genes and a typical yellow popcorn line as the recurrent parent (Fig. 1). The *Pl* and *B* transcription factors conferred colour to the seed pericarp of the newly developed lines (Fig. 2). The SSRs, *nc009* and *umc1776* being part of the *Pl1 and B1 loci* respectively, were polymorphic between the coloured and the colourless plants (Fig. 3) allowing us to select the *B1b1 Pl1pl1* heterozygotes in the backcrossing recurrent selection.

In order to select the best anthocyanin accumulator popcorn line, we also quantified the pigment content in each of the 21 near isogenic lines developed in the Landriano experimental field and the anthocyanin levels ranged between 10.7 mg/100g and 66.44 mg/100g (data not shown). All the analysis hereinafter described were performed on the line that showed the highest anthocyanin content, using as a control a typical yellow popcorn line: both lines were developed in the field of Landriano.

The control yellow line, as expected, showed no detectable anthocyanin content (data not shown).

The ear, the seeds and the flakes of the colourless and coloured popcorn lines can be seen in Fig. 2: the white amylaceous popping part showed no difference between the two lines, while the anthocyanins accumulation, determined by the *B1-Pl1* genes, was specific to the pericarp, that appeared yellow in the colourless line and almost black in the coloured one.

Qualitative features

We tested the quality of the coloured popcorn line by the measurements of 4 important agronomic/qualitative parameters: the germinability of the seeds, the popping percentage of popcorn seeds, the flake volume and the popping expansion volume (PEV) (Table 1).

The data obtained showed that while the percentage of germination was significantly lower in coloured compared to the colourless popcorn line (90% vs

100%), for all the other parameters considered no statistical differences were found between coloured and colourless popcorn (Table1).

Nutritional properties

In order to find out possible changes in the anthocyanin molecules caused by the microwave treatment, we performed quantitative and qualitative analysis on the anthocyanin content before and after popping.

In Online Resource 1 the anthocyanin content in coloured seeds before and after the microwave treatment showed that these pigments are subject to a statistically significant decrease of about 46% .

To check if this quantitative reduction affected some specific anthocyanin molecules or was a general decrease, we analysed the TLC (Online Resource 2) and HPLC results (Fig. 4).

The picture of the TLC plate taken with visible light (Online Resource 2A), showed that the cyanidin is the most abundant anthocyanin in the coloured popcorn line maize, both before and after the microwave treatment (Online Resource 2A). It is also possible to see a small amount of pelargonidin in both the samples, and the relative ratio between cyanidin and pelargonidin seems to remain the same in the two samples (Online Resource 2).

These were confirmed by HPLC analysis. The chromatogram of the seed pericarp showed a high peak identified as cyanidin, and a smaller one as pelargonidin (Fig. 4). The peaks of the flake pericarp were qualitatively the same, only quantitatively smaller (Fig. 4). Interestingly the UV illumination picture of the TLC plate showed another unidentified spot (Online Resource 2), not visible in the chromatograms (Fig. 4). More accurate analyses are needed for its identification.

Finally the anthocyanins present in the pericarp of the colourless and coloured seeds, before and after popping were extracted and their antioxidant ability was quantified.

The comparison of the curves of the 2 colourless samples (seeds and flakes), in which no anthocyanins were detected, showed no difference in radical scavenging

ability (Online Resource 3). Nearly the same pattern was found by the comparison of the 2 coloured samples (Online Resource 3), in which the anthocyanin concentration was equalized. The antioxidant ability of the coloured extracts is clearly higher with respect to the colourless ones, as expected.

Panel Test/ consumer test

We tested the acceptability of the new coloured popcorn in comparison with a commercial yellow one, in a blindfold test on a consumer sample of 67 subject, randomly chosen. Both popcorn flakes were tested without salt and dressing.
The acceptability scores were 6.61 for the commercial yellow popcorn and 6.75 for the coloured one, statistical analysis showed no difference between these values (Online Resource 4).

Discussion

The aim of this work was to develop a popcorn line with a high content in anthocyanins to make this popular snack a healthier functional food. The selection was helped by the use of the *nc009* and *umc1778* SSRs, polymorphic between the coloured and the colourless popcorn lines (Fig. 3). In fact the DNA analysis of the seedlings, coming from each ear, is an accurate and faster procedure to select the individuals that will produce a coloured progeny. From this breeding scheme we obtained 21 NILs (Nearly Isogenic Lines) that accumulated the pigments in the outer layer of the kernel , the pericarp (Fig. 2), in the cob and anthers.
To select the popcorn line with the highest anthocyanins content, we thus quantified the anthocyanins level in each ear. The best pigment accumulator displayed a level of anthocyanins of 66.44 mg/100g (Online Resource 1) so this line appeared to be a good candidate to become a functional food. This line was further analysed to quantify some traits fundamental in the determination of its quality as a popcorn line. The coloured popcorn line showed a germination percentage significantly lower (by 10%) in comparison to the control yellow line, maybe due to the typical inbreeding depression arising during the development of

a line. On the other hand the popping ability of the coloured line was comparable to that of the commercial yellow popcorn line, and also the most important parameter to be considered in determining the quality of a popcorn line, i.e. the expansion capacity of the kernel during its transformation into popped flake, (PEV) showed a value (9.8) not significantly different from that of the control (Table 1). This is a very encouraging result even compared to those of Babu et al. (2006) who found values ranging from 2.5 to 18.4. Also the flake volume of the coloured popcorn of about 0.7 cm^3 was the same as that of the colourless control, although Babu et al. (2006) found values ranging from 0.96 to 3.1 cm^3 and Soylu and Tekkanat (2007) found values from 2.75 to 6.83 cm^3. This difference could be explained by the lower weight of the seeds obtained in our experimental field conditions. In fact we measured an average weight per seed of 11.8 mg while Soylu and Tekkanat (2007) reported an average weight of 14.5 mg. Furthermore the popcorn weights recorded by Soylu and Tekkanat (2007) were obtained using a hot-air popcorn popper while we used a microwave machine.

However the fact that the flake volume of the coloured popcorn was the same as that of the colourless control is an important result: the value of the flake volume could compromise the success of the new coloured variety because the consumers preferences are towards larger flakes (Ziegler et al. 1984). Another important aspect is the wide confidence intervals found for each parameter measured on the coloured line, compared to the control. This variability which still characterizes the new coloured popcorn line could be reduced by further cycles of back-crossing which could thus stabilize the values of both kernels' and popped flakes' volume.

The presence of the anthocyanins in a very popular snack could help to introduce or to increase the consumption of antioxidant and healthy compounds in the dietary habits of many people. Therefore it would be useful to establish whether and to what extent the thermal treatment of the microwave is able to modify or to degrade these pigments.

Previous work showed that both temperature and food processing can affect the stability and content of the anthocyanins in the final products_(Giusti and Wrolstad 2003; Jackman and Smith 1996).

For example elderberry anthocyanin contents decrease by about 50% after heating (Sadilova et al. 2006), and a similar loss was found also in raspberry purees (Ochoa et al. 1999). Besides, a baking treatment of muffins enriched with red raspberry juice significantly decreased the total phenolics present in these popovers (Rosales-Soto et al. 2012).

However Rodrigues et al. (2009) have also shown that the anthocyanin amount present in onion landraces was not modified by mild microwave cooking. The analyses performed on coloured popcorn showed a decrease in the anthocyanins content of about 46% (Online Resource 1) caused by the microwave treatment, probably because the treatment at 800W for more than 2 minutes cannot be considered mild cooking.

The content of 66.44 mg of anthocyanins per 100g of coloured popcorn seeds and the subsequent 46% reduction caused by the microwave treatment may appear to result in a low level of anthocyanins in comparison with berries (i.e. blueberries from 25 to 495 mg/100g (Mazza and Miniati 1993), but for a more objective evaluation it is necessary to take into account the consumption per capita. Indeed while Americans consume 52 quarts of popped corn per year according to the Agricultural Marketing Resource Centre (http://www.agmrc.org/commodities__products/grains__oilseeds/corn_grain/popcorn_profile.cfm), they consumed only 0.5 pounds of blueberries or 6.2 pounds of strawberries in 2007 (USDA ERS-Fresh fruit farm weight http://www.ers.usda.gov/data/foodconsumption/FoodAvailspreadsheets.htm#fruitfr). Furthermore also availability and perishability have to be considered: pop maize can be stored as seeds for years, while berries have a very short shelf life. So taking into account all these aspects, the anthocyanins content achieved in coloured popcorn can be seen as a good result, particularly when we also consider that continued genetic breeding and plant research can help to further increase this value in future.

A key point in the production of a functional snack is whether the microwave treatment can change the anthocyanin molecule structure and consequently their antioxidant capability. The TLC and HPLC analyses performed on the pericarp of

the coloured kernels and on the coloured popcorn after microwave treatment found that the relative ratio between the 2 most abundant anthocyanins (cyanidin and pelargonidin) remained roughly the same. From the TLC plate in fact the spots classified as cyanidin and pelargonidin according to the standards did not present clear differences before and after microwave heating (Online Resource 2). This result was also confirmed by the HPLC chromatograms: the peaks for the flakes were only quantitatively smaller and not qualitatively different (Fig. 4). So the microwave treatment determined a loss of the anthocyanin content but not a structural modification of these pigments.

Recent studies have shown that thermal treatment was able to increase the antioxidant activity of various vegetables, among them sweet corn. This effect has been explained by considering the Maillard reaction products (Dewanto et al. 2002; Nindo et al. 2003). For this reason only the pericarp powders were used in the radical scavenging test: to avoid the interfering effect of the Maillard reaction products we have eliminated the endosperm rich in starch. In fact the curves of the colourless unpopped and popped samples showed a very similar pattern and no new antioxidant compounds were derived by the microwave treatment. To better dissect the possible effect of the microwave treatment on the antioxidant power of the coloured popcorn we compared the DPPH scavenging ability of equal concentrations of anthocyanins obtained from unpopped and popped coloured seeds. The graph in the Online Resource 3 showed no substantial differences between the radical scavenging ability of the colored unpopped and popped samples, confirming that the microwave heating did not modify the integrity of anthocyanin molecules and their antioxidant activity.

We performed this analysis on samples whose anthocyanins concentration was previously equalized because we would like to compare the antioxidant activity and not to have the absolute values and also to overcome a possible technical problem. In fact the wavelength of 516 nm used to assess the DPPH discoloration through the spectrophotometer is close to the 530 nm used to measure the anthocyanin concentration, so the discoloration readings could be perturbed by

the presence of the pigments. Using an equal amount of the anthocyanins for both the samples was the solution we used to overcome this problem.

The curves obtained by the analysis of coloured popcorn showed a higher capacity in the DPPH scavenging compared with the colourless samples. This result was not surprising because of the renowned antioxidant capacity of the anthocyanins and of the flavonoids in general (Nijveldt et al. 2001; Urquiaga et al. 2000). The antioxidant activity of anthocyanins is due to the ability of these molecules to scavenge free radicals by donation of phenolic hydrogen atoms (Chen et al. 1996). This property has long been studied not only for the important roles played within plants but also for therapeutic purposes. In fact the antioxidant power of anthocyanins has been related to anti-inflammatory and anticancerogenic properties, to cardiovascular disease prevention, to obesity and diabetes control (Tsuda 2012). In a comparative work regarding the chemoprotective effect of different anthocyanin-rich extracts from purple corn, chokeberry, bilberry, purple carrot, grape, radish and elderberry it was resulted that purple corn exhibit the best inhibition of colon cancer cell (HT29 human cell line) proliferation (Jing et al 2008). In particular these authors reported that anthocyanin chemical structure affected chemoprotection, suggesting that the cyanidin glucoside anthocyanins may be effective chemoprotective compounds while pelargonidin exerted the lowest effect (Jing et al 2008). Of course further works will be necessary to assess the bioavailability of the anthocyanins in this food matrix and to characterise the anthocyanins chemical structure (in particular acylation and glicosilation) pre and post microwave treatment.

Finally, even if after the microwave treatment nearly one half of the original pigment amount was lost (Online Resource 1), the new coloured snack still retained an appreciable level of antioxidant activity (Online Resource 3) and thus its functional characteristics.

Nevertheless when a new product is going to enter the market, it is important to test the consumers' rating of acceptability. The mean values obtained were of 6.61±0.34 for the traditional yellow popcorn and 6.75±0.36 of the coloured one (Online Resource 4). These appreciation degrees may not seem to be very high on

a scale starting from 1, the lowest, to 10, the highest. However we must consider that the subjects were asked to pass judgment about a snack without dressing, an unusual procedure chosen to maximise the perception of any possible difference in taste between the colourless and the coloured popcorn. Thus the lack of difference between the acceptability rating for the 2 types of popcorn is very important. This result suggested that the consumer will probably choose the coloured popcorn because it has the same taste as the traditional colourless one, but it is healthier.

Acknowledgments

We wish to thank Dr. Davide Reginelli for his hard work in the field.

Figures and Tables

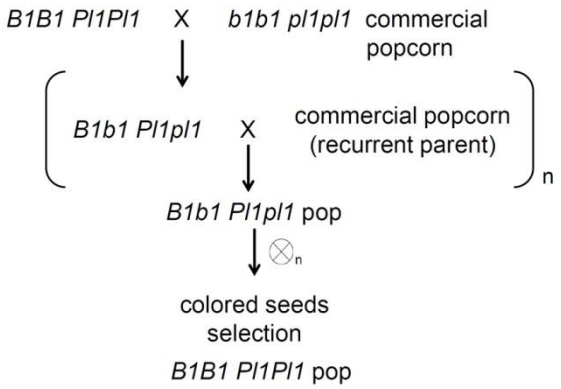


Figure 1. Recurrent Selection Scheme: after the first cross between the line B1Pl1, source of the regulatory biosynthetic genes and the commercial popcorn, the lines heterozygous for the *B- Pl-* genes and with the highest anthocyanin content were selected for backcrossing. The 21 resulting lines were further selected for the highest level of anthocyanins and underwent some cycles of self-pollination

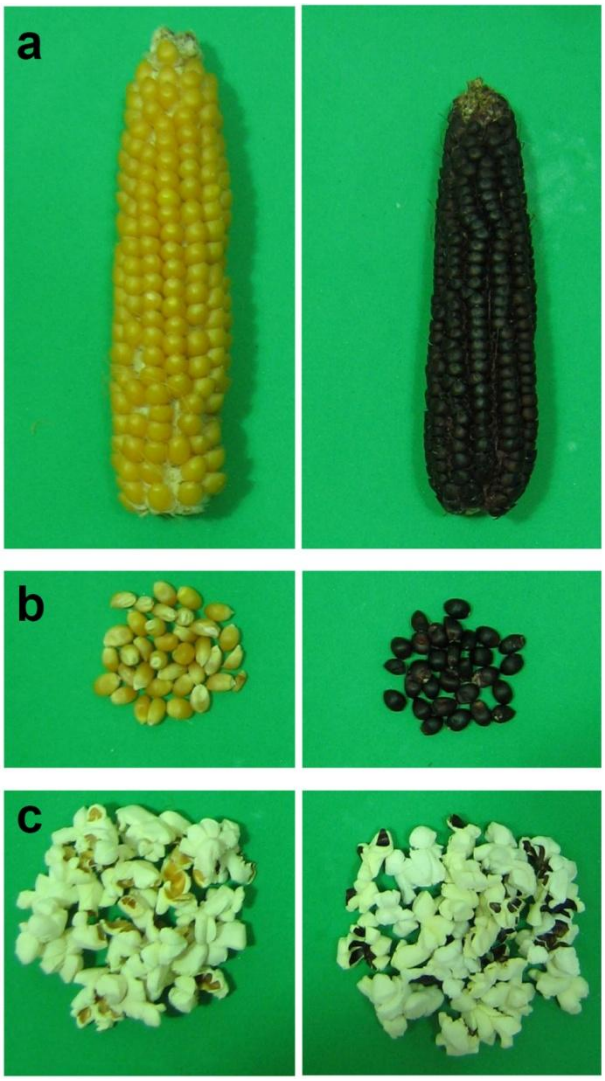


Figure 2. Phenotype of ear (A), kernels (B) and flakes (C) of the new coloured popcorn line (right) and of the traditional colourless one (left)

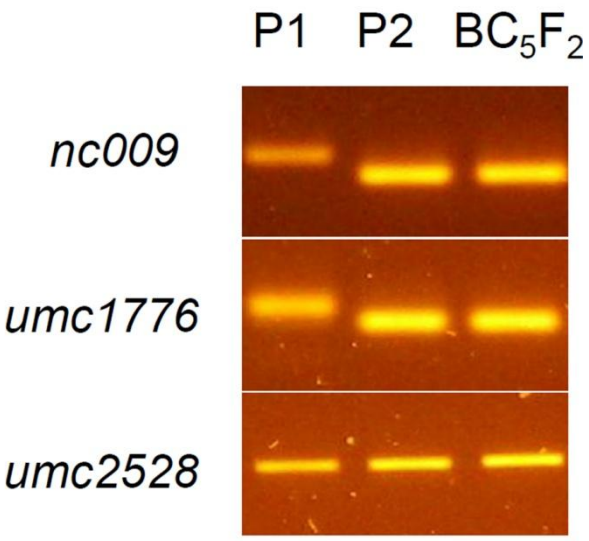


Figure 3. Molecular Assisted Selection: two of the three SSRs used were polymorphic between the coloured and the colourless popcorn lines. The *nc009* is the SSR part of the *Pl1* gene and the *umc1776* SSR is part of the *B1* gene. The *umc2528* SSR, associated to the *R* gene not involved in the selection, did not show polymorphism between the two popcorn lines. The heterozygous individuals were easily detected and selected to carry on the breeding selection. P1 parent 1; P2 parent 2; BC back crossing

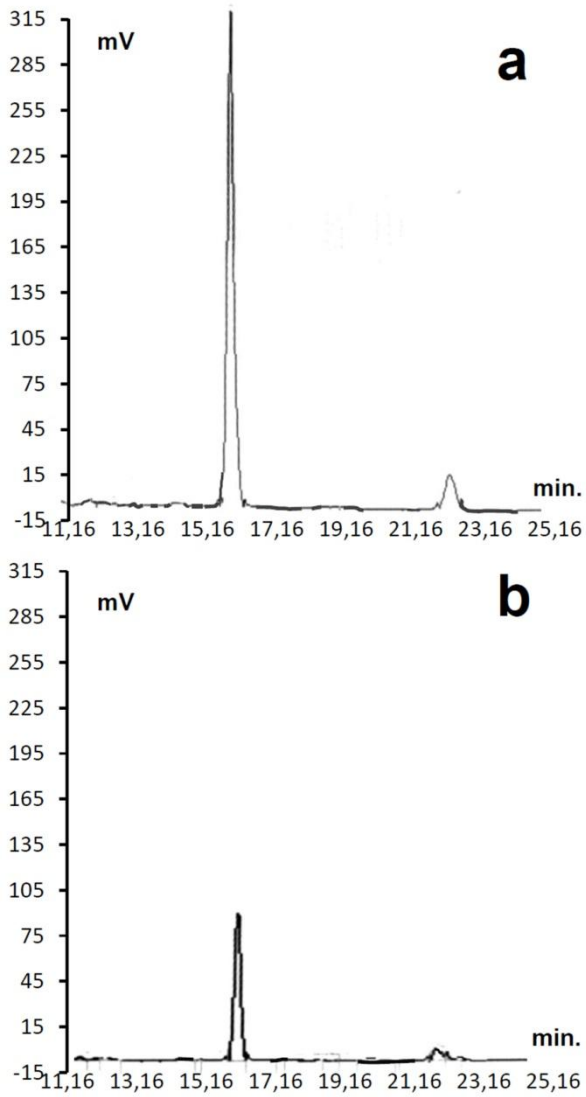


Figure 4. HPLC chromatograms of the anthocyanins extracted from the pericarp of coloured seed (A) and of popcorn flakes (B). The first and the highest peak corresponds to the cyanidin, while the second and smallest one to the pelargonidin, according to the standards chromatogram.

Table 1. Values of the traits measured in colourless and coloured lines. Confidence Interval at 95% are shown

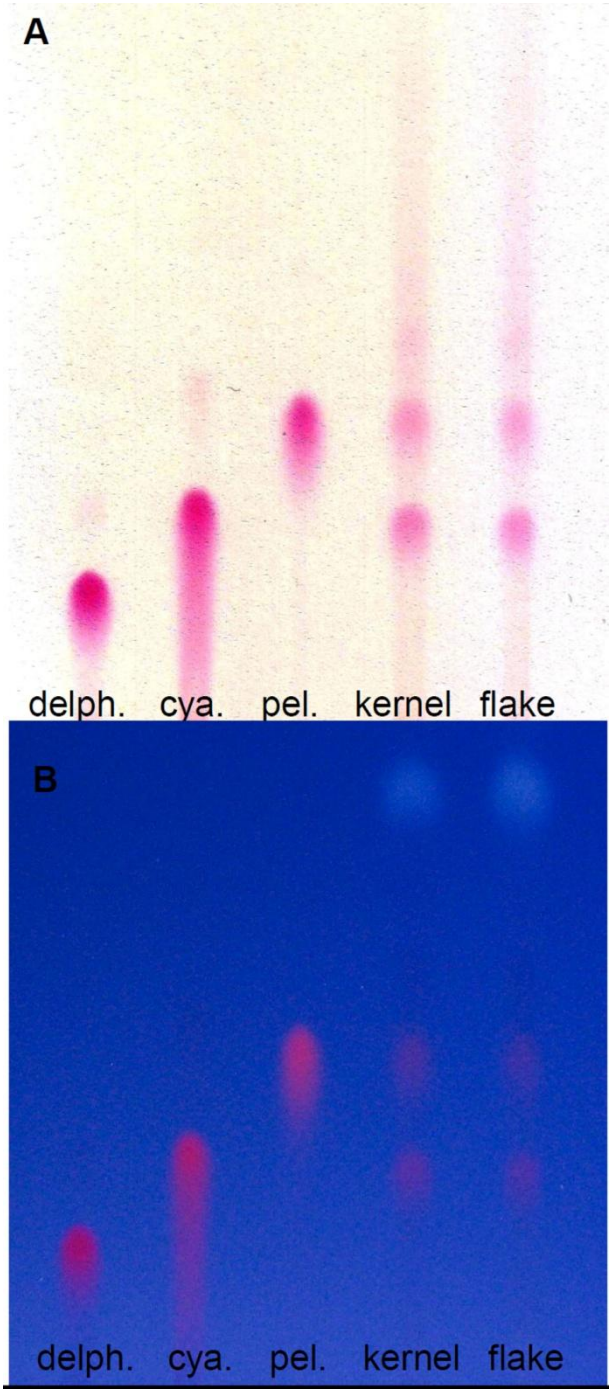
	germinating ability (%)	popping ability (%)	flake volume (cm^3)	PEV[1]
colourless	100	78.79 ± 8.09	0.739 ± 0.069	8.040
coloured	90 ± 9.11	88.12 ± 6.34	0.759 ± 0.235	9.805

[1] Popping Expansion Volume

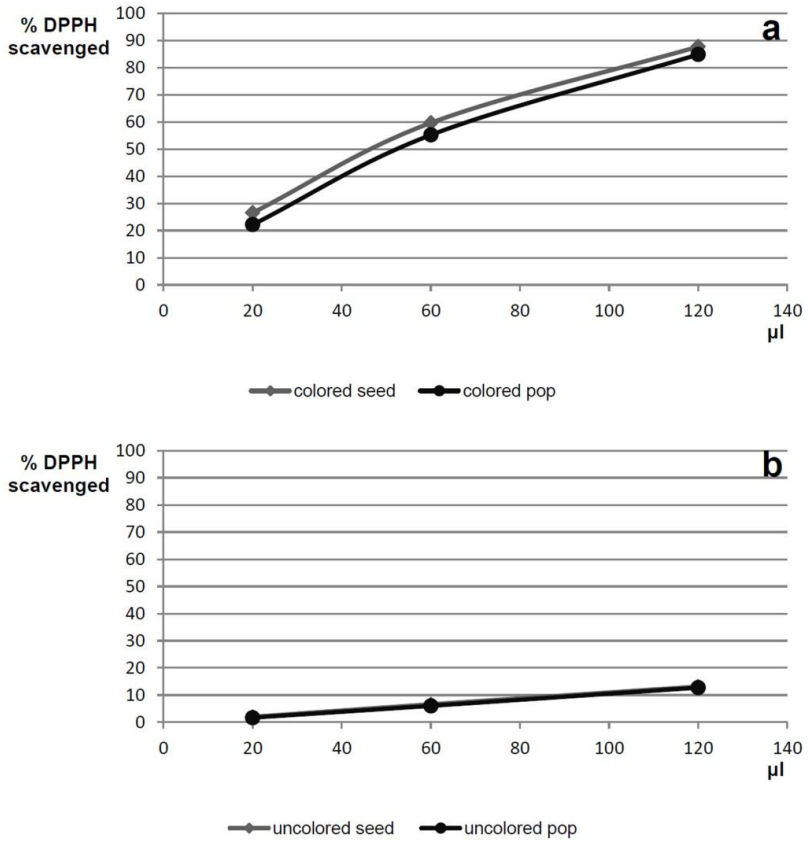
Online Resource 1. Effect of the microwave treatment on the anthocyanin content on both the colourless and coloured seeds and popcorn flakes. Confidence Interval at 95% are shown

	anthocyanins content (mg/100g) before microwave	anthocyanins content (mg/100g) after microwave	reduction %
colourless line	N.D.[1]	N.D.[1]	-
coloured line	66.44 ± 4.95	35.83 ± 4.40	46.07

[1] Not Detectable



Online Resource 2. Pictures of the TLC plate taken under visible (A) or UV light (B). The spots represent from left to right: delphinidin, cyanidin and pelargonidin standards, the anthocyanin extracts from the kernels and from the flakes after the microwave treatment.



Online Resource 3. Comparison of the antioxidant ability in the DPPH radical scavenging test of seeds vs flakes coloured (a) and colourless (b).

Online Resource 4. Panel test. Results obtained comparing coloured popcorn Vs the traditional colourless one. Confidential Interval at 95% are shown.

	colourless	coloured
mean	6.61 ± 0.34	6.75 ± 0.36
median	7	7
mode	7	7

References

Astadi IR, Astuti M, Santoso U, Nugraheni PS (2009) In vitro antioxidant activity of anthocyanins of black soybean seed coat in human low density lipoprotein (LDL). Food Chem 112: 659-663

Babu R, Nair SK, Kumar A, Rao HS, Verma P, Gahalain A, Singh IS, Gupta HS (2006) Mapping QTLs for popping ability in a popcorn X flint corn cross. Theor Appl Genet 112:1392-1399

Bodeau JP, Walbot V (1992) Regulated transcription of the maize *Bronze-2* promoter in electroporated protoplasts requires the *C1* and *R* gene products. Mo1 Gen Genet 233:379-387

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. Lebensm Wiss Technol 28:25-30

Cevallos-Casals BA, Cisneros-Zevallos L (2003) Stoichiometric and kinetic studies of phenolic antioxidants from Andean purple corn and red-fleshed sweetpotato. J Agric Food Chem 51:3313-3319

Ceylan M, Karababa E (2001) Comparison of sensory properties of popcorn from various types and sizes of kernel. J Sci Food Agric 82:127-133

Chandler VL, Radicella JP, Robbins Chen JC, Turks D (1989) Two regulatory genes of the maize anthocyanin pathway are homologous: isolation of *B* utilizing *R* genomic sequences. Plant Cell 1:1175-1183.

Chen ZY, Chan PT, Ho KY, Fung KP, Wang J (1996) Antioxidant activity of natural flavonoids is governed by number and location of their aromatic hydroxyl groups. Chem Phys Lipids 79:157-163

de Pascual-Teresa S, Sanchez-Ballesta MT (2008) Anthocyanins: from plant to health. Phytochem Rev 7:281-299

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19-21

Dewanto V, Wu X, Liu RH (2002) Processed sweet corn has higher antioxidant activity. J Agric Food Chem 50:4959-4964

Dooner HK, Robbins TP, Jorgensen RA (1991) Genetic and developmental control of anthocyanin biosynthesis. Annu Rev Genet 25:173-199

Escribano-Bailón MT, Santos-Buelga C, Rivas-Gonzalo JC (2004) Anthocyanins in cereals. J Chromatogr A 1054:129-141

Gaut BS (2001) Patterns of chromosomal duplication in maize and their implications for comparative maps of the grasses. Genom Res 11: 55-66

Giusti MM, Wrolstad RE (2003) Acylated anthocyanins from edible sources and their applications in food systems. Biochem Eng J 14:217-225

Goodman CD, Casati P, Walbot V (2004) A multidrug resistance associated protein involved in anthocyanin transport in *Zea mays*. Plant Cell 16:1812-1826

Grobman A, Bonavia D, Dillehay TD, Piperno DR, Iriarte J, Holst I (2012) Preceramic maize from Paredones and Huaca Prieta, Peru. PNAS 109:1755-1759

Hu FL, Lu RL, Huang B, Ming L (2004) Free radical scavenging activity of extracts prepared from fresh leaves of selected Chinese medicinal plants. Fitoterapia 75: 14-23

Jackman RL, Smith JL (1996) Anthocyanin and betalains. In: Hendry GAF and Houghton JD (ed) Natural food colorants. Blackie Academic and Professional, Glasgow, pp. 249-309

Jing P, Bomser JA, Schwartzt SJ, He J, Magnuson BA, Giusti MM (2008) Structure-Function Relationships of Anthocyanins from Various Anthocyanin-Rich Extracts on the Inhibition of Colon Cancer Cell Growth. J Agric Food Chem 56:9391-9398

Karababa E (2006) Physical properties of popcorn kernels. J Food Eng 72:100-107.

Leong LP, Shui G (2002) An investigation of antioxidant capacity of fruits in Singapore markets. Food Chem 76:69-75

Lippi G, Franchini M, Favaloro EJ, Targher G (2010a) Moderate red wine consumption and cardiovascular disease risk: beyond the "French paradox". Semin Thromb Hemost 36:59-70

Lippi G, Franchini M, Guidi GC (2010b) Red wine and cardiovascular health: The "French Paradox" revisited. Int J Wine Res 2:1-7

Martin C, Butelli E, Petroni K, Tonelli C (2011) How can research on plants contribute to promoting human health? The Plant Cell 23:1685-1699

Mazza G and Miniati E (1993) Anthocyanins in fruits, vegetables, and grains. CRC Press, Boca Raton, FL

Nakatani N, Fukuda H, Fuwa H (1979) Major anthocyanin of Bolivian purple corn (*Zea mays* L.). Agric Biol Chem 43:389-391

Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA (2001) Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 74:418-25

Nindo CI, Sun T, Wang SW, Tang J, Powers JR (2003) Evaluation of drying technologies for retention of physical quality and antioxidants in asparagus (*Asparagus officinalis* L.). Lebensm Wiss Technol 36:507-516

Ochoa MR, Kesseler AG, Vullioud MB, Lozano JE (1999) Physical and chemical characteristics of raspberry pulp: storage effect on composition and color. Lebensm-Wiss Technol 32:149-153

Pilu R, Piazza P, Petroni K, Ronchi A, Martin C, Tonelli C (2003) *pl-bol3*, a complex allele of the anthocyanin regulatory *pl1* locus that arose in a naturally occurring maize population. Plant J 36:510-521

Pilu R (2011) Paramutation: Just a curiosity or fine tuning of gene expression in the next generation? Curr Genomics 12:298-306.

Pilu R, Bucci A, Casella L, Lago C, Cerino Badone F, Cassani E, Landoni M (2012) A quantitative trait locus involved in maize yield is tightly associated to the *r1* gene on the long arm of chromosome. Mol Breeding 30:799-807

Robbins WA, Ashman RB (1984) Parent-offspring popping expansion correlations in progeny of dent corn × popcorn and flint corn × popcorn crosses. Crop Sci 24:119-121

Rodrigues AS, Pérez-Gregorio MR, García-Falcón MS, Simal-Gándara J (2009) Effect of curing and cooking on flavonols and anthocyanins in traditional varieties of onion bulbs. Food Research International 42:1331-1336

Rosales-Soto MU, Powers JR, Alldredge JR (2012) Effect of mixing time, freeze-drying and baking on phenolics, anthocyanins and antioxidant capacity of raspberry juice during processing of muffins. J Sci Food Agric 92:1511-1518

Sadilova E, Stintzing FC, Carle R (2006) Thermal degradation of acylated and non acylated anthocyanins. J Food Sci 71:C504-C512

Schwarz M, Hillebrand S, Habben S, Degenhardt A, Winterhalter P (2003) Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins. Biochem Eng J 14:179-189

Song A, Eckhoff SR, Paulsen M, Litchfield JB (1991) Effects of kernel size and genotype on popcorn popping volume and number of unpopped kernels. Cereal Chem 68:464-466

Soylu S, Tekkanat A (2007) Interactions amongst kernel properties and expansion volume in various popcorn genotypes. J Food Eng 80:336-341

Tsuda T (2012) Dietary anthocyanin-rich plants: biochemical basis and recent progress in health benefits studies. Mol. Nutr. Food Res. 56, 159-170.

Urquiaga I, Leighton F (2000) Plant polyphenol antioxidants and oxidative stress. Biol Res 33:55-64

Virgili F, Marino M (2008) Regulation of cellular signals from nutritional molecules: A specific role for phytochemicals, beyond antioxidant activity. Free Radic Biol Med 45:1205-1216

WHO Noncommunicable diseases country profiles 2011 ISBN 978 92 4 150228 3 http://www.who.int/topics/chronic_diseases/en/

Yang Z, Zhai W (2010) Identification and antioxidant activity of anthocyanins extracted from the seed and cob of purple corn (*Zea mays* L.). Innov Food Sci Emerg 11:169-176

Ziegler KE (2001) Popcorn. In: Hallauer AR (ed) Specialty corns, 2nd edn. CRC Press, Boca Raton FL, pp 199-234

Ziegler KE, Ashman RB, White GM, Wysong DS (1984) Popcorn production and marketing. National Corn Handbook, NCH-5, Purdue University, West Lafayette, Indiana.

Development and study of a maize cultivar rich in anthocyanins: coloured polenta, a new functional food

Chiara Lago[1], Elena Cassani[1], Cecilia Zanzi[1], Michela Landoni[2], Riccardo[1] Trovato[1], Roberto Pilu[1].

[1] Dipartimento di Scienze Agrarie e Ambientali - Produzione, Territorio, Agroenergia, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy

[2] Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

This is a pre-copy-editing, author-produced of an article accepted for publication in PLANT BREEDING.

Lago C, Cassani E, Zanzi C, Landoni M, Trovato R, Pilu R. Development and study of a maize cultivar rich in anthocyanins: coloured polenta, a new functional food. DOI: 10.1111/pbr.12153.

The definitive publisher-authenticated version is available online at:
http://onlinelibrary.wiley.com/journal/10.1111/%28ISSN%291439-0523/issues

Abstract

Among the phytonutrients, anthocyanins have been extensively studied in several different vegetables, because of their antioxidant power, the characteristic supposedly responsible for their capacity for chronic disease prevention. Anthocyanins can also be synthesized in maize even though in Europe the colourless varieties have always been preferred. The aim of this study was to develop and characterize a new polenta variety of maize rich in anthocyanins, bred by a recurrent selection scheme, to increase the antioxidant power of this food. The recurrent selection was based on the anthocyanin content and other specific traits of the kernel. The coloured polenta obtained was analysed by TLC (Thin Layer Chromatography) analysis and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging ability, before and after cooking. The results obtained showed that even though cooking reduced the anthocyanin content by about 22 %, the remaining anthocyanins exhibited a good antioxidant capacity compared to the colourless control. Furthermore our data showed that the anthocyanin content did not alter the taste of the coloured polenta, and no difference in flavour was perceived between coloured and uncoloured polenta.

Key words: *Zea mays* – breeding – polenta – anthocyanins - functional food

Introduction

The main cereal grain cultivated throughout the world is maize (Zea mays L. ssp. mays), maybe because of its versatility (Food and Agriculture Organization of the United Nations, Crops Production, 2009; Zeppa et al. 2012). It can be used as feed for livestock, forage, silage and grain, but also for biofuel and for industrial uses. However human nutrition remains one of the main uses, determining the selection of varieties for producing many locally typical cornmeals 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). It is made by constantly stirring the cornmeal with salted water, throughout a slow cooking process lasting up to 1 hour, when this porridge-like dish is ready. Different kinds of polenta exist on the basis of the maize variety and of the type of flour milling. Historically Italian polenta maize was obtained from landraces, fitted to the different microclimates peculiar to the several Italian agroclimatic areas (Brandolini and Brandolini 2009). Traditionally the landraces used in Italy for human feeding have a flint or semi-flint kernel texture (Brandolini and Brandolini 2009). Kernels of flint corn mostly have hard, glassy endosperms with smooth, hard seed coats (pericarps) (Dickerson 2003).

Corn flour composition is well defined, it is mainly composed by starch (80%), proteins (10-15%) and lipids (5%) (Panzeri et al. 2011). However, there have been few specific studies on polenta: it is known that polenta can be a good source of iron and phosphorus, and also of carotenoids, most of which are provitamins A (Venturelli et al. 1990; Brandolini and Brandolini 2009; Rodriguez-Amaya et al. 2008). Polenta was generally considered a food for the poor: in 1800 it was the emblem for the humble and rough plebeians, who were forced by wars and famine to consume it as their only dish, which led to their getting sick of pellagra, a dietary deficiency of niacin and tryptophan (Sebrell 1981). Today a key challenge for breeders is the improvement of the nutritional composition of corn grain and of polenta in order to enhance its nutritional value (Berardo et al. 2004). A starting point could be to increase the antioxidant power: functional foods, rich in antioxidant compounds are currently attracting wide interest.

According to several studies a direct antioxidant effect played by many food phytonutrients and other metabolites seems to be able to prevent several chronic diseases (Virgili and Marino 2008). Chronic diseases are non-communicable diseases characterized by a long duration and a generally slow progression, that have become the biggest cause of death worldwide. They include cardiovascular diseases, cancers, respiratory diseases, diabetes and obesity (WHO Non communicable diseases country profiles 2011).

An important source of hydrophilic dietary antioxidants in maize could be the flavonoids. In fact corn is able to accumulate anthocyanins, a sub-group of the flavonoids (Nakatani et al. 1979; de Pascual-Teresa et al. 2002; Escribano-Bailon et al. 2004).

Anthocyanins have been intensively studied in several different vegetables, because of their antioxidant power, the putative characteristic responsible for many health benefits. They have proven to be able to lower LDL-cholesterol levels (Castilla et al. 2008; Tsuda 2012), to significantly reduce the risk of death from heart disease (Rissanen et al. 2003; Toufektsian et al. 2008; Tsuda 2012), to fight obesity (Seymour et al. 2009; Titta et al. 2010; Peng et al. 2011; Tsuda 2012) and diabetes (Tsuda 2008; Prior et al. 2008; DeFuria et al. 2009; Tsuda 2012), to improve visual function (Matsumoto et al. 2005; Iwasaki-Kurashige et al. 2006) and to prevent neurodegenerative diseases (Goyarzu et al. 2004; Lau et al. 2007; Shukitt-Hale et al. 2008; Tsuda 2012). The corn genotypes displaying the anthocyanin colorations ranging from red to dark blue are grown widely by traditional farmers in Central and South America, even though the majority of maize varieties including those with white or yellow grains have the genetic information for the anthocyanin biosynthetic pathway. Two multigene families are required for the regulation of the anthocyanin pathway: the r1/b1family belonging to the class of bHLH transcription factors and the c1/pl1/p1 belonging to the class of MYB transcription factors (Chandler et al. 1989; Dooner et al. 1991; Pilu et al. 2003). A member of each family must be present and active in the dominant form to activate anthocyanin biosynthesis and according to the combination of alleles of the regulatory genes, the synthesis will be active in different plant tissues. The

typical dark blue colour of some tropical maize seeds is obtained by the allelic combination of B/Pl regulatory genes, able to activate mainly the cyanidin-3-glucoside synthesis in the pericarp (Nakatani et al. 1979; de Pascual-Teresa et al. 2002; Escribano-Bailon et al. 2004) but also in the plant (Chandler et al. 1989; Bodeau and Walbot 1992; Gaut 2001; Pilu et al. 2003; Lago et al. 2012).

The nutritional value of maize and polenta can thus be enhanced by increasing the level of anthocyanin content: in this way the new colored polenta could be considered a functional food.

This study describes the development and characterization of a new polenta maize line able to accumulate anthocyanins and well fitted to growing in the northern region of Italy where polenta is a very popular dish. This line (derived from the 'Scagliolo' cultivar, able to grow at 635m in the Valsassina area) allows the production of a coloured polenta, thus increasing the amount of antioxidant molecules ingested by people. In this way not only the nutritional and commercial value on the market of this new coloured polenta maize line will be enhanced but it could also promote the project of prevention of chronic diseases conducted by international health organizations.

Materials and Methods

Plant material

A recurrent breeding scheme was planned and applied to obtain a new coloured polenta maize line in the experimental field of the University of Milan located in Landriano (PV, Italy). The 'Scagliolo' variety (from Carenno LC, VA1210) was chosen as the recurrent parent for the 5 cycles of backcrossing. The sources of the dominant anthocyanin biosynthesis regulation were the genes Booster1 (B) and Purple plant1 (Pl) in the homozygous state, which determine pericarp and plant colour. After the first cross (B1B1Pl1Pl1 X scagliolo) where we used the inbred line B1B1Pl1Pl1 as male we backcrossed five times using as male the pollen collected and mixed from about 20 male inflorescence of "Scagliolo" variety

randomly selected. In every cycle of BC we selected the ears (about ten out of 100) with the higher anthocyanin content for the next BC cycle. Hence we selfed three times the best material and so doing we obtained the four sublines homozygous for the B and Pl genes. The heterozygous plants (Pl1pl1 B1b1) coming from backcrossing and those from self-pollination were selected according to the anthocyanins content and by using Marker Assisted Selection (MAS).

Molecular Marker Assisted Selection

The MAS was performed on the DNA extracted from the leaves of parental (P1 and P2) and progenies' plants, as previously described (Dellaporta et al. 1983). The umc1014 SSR molecular marker (5'GAAAGTCGATCGAGAGACCCTG3'/5'CCCTCTCTTCACCCCTTCCTT3') part of the pl1 gene located on chromosome 6 and the umc1024 SSR molecular marker (5'CCTTTTTCGCCTCGCTTTTTAT/5'TCGTCGTCTCCAATCATACGTG3') associated to the b1gene on the short arm of chromosome 2 from MaizeGDB (http://www.maizeg db.org/ssr.php) were used.

The umc2528 SSR (5'CTCATCAACATGCAAAGGACGTAG3'/5'ATTCAAATGCCTCTAAGCTAGCCG3') was also used: it is associated to the r gene on chromosome 10 not directly involved in the selection. Polymerase Chain Reactions (PCR) and gel running conditions were carried out as described in the SSR Methods Manual by MaizeGDB (http://www.maizegdb.org/documentation/maizemap/ssr_protocols.php).

Material Sampling

For all genotypes tested (4 coloured sublines and the colourless parental "Scagliolo") in the 2011 field season about 200 plants were grown in adjacent rows, under the same agronomic conditions, in the experimental field of the

University of Milan, Italy (N 45°18′, E 9°15). These plants were selfed (using paper bags) and then harvested at the same time at the end of the season.
About 100 ears were shelled and the seeds obtained mixed to create a single bulk used for the determination of mean seed weight, for the germination test and to make polenta. For anthocyanins quantification we used 4 unshelled ears (for each genotype) randomly selected from the remaining harvest.

Mean seed weight

Forty seeds of each new coloured sub-line and of the yellow 'Scagliolo' control variety were weighed. The mean weight and the confidence intervals at 95% were calculated.

Germination test

Fifteen seeds of each coloured sub-line and of the yellow 'Scagliolo' control variety were germinated in Plexiglas boxes on filter paper imbibed with water, in darkness for 7 days at 24°C to check the germination percentage.

Quantification of anthocyanins

Four kernels from each ear were cleaned from the glumes, ground down to a fine powder with a ball mill and pooled together within each line. To quantify the anthocyanins content of each sub-line, 5 mg of the powder were boiled with 100 µl of distilled water for 30 minutes. Then the samples were left overnight in agitation with 1 ml of the extraction buffer (1% HCl, 95% ethanol). After collecting the supernatants with a centrifugation at 13000rpm for 10 minutes, a second extraction cycle of 2 hours in 500 µl of extraction buffer was made. Finally the supernatants were collected together and after a centrifugation at 13000rpm for 30 minutes, their absorbance was determined spectrophotometrically at □□□530 nm. The anthocyanin amount was calculated as cyanidin-3-glucoside equivalents (molar extinction coefficient (e) 26900 L m-1mol-1, MW 449.2) for 100 g of dry flour. The analyses were conducted on four ears randomly selected for each line and the

confidence interval (C.I.) at 95% was calculated. The same procedure was carried out on a 'Scagliolo' yellow maize variety, grown in the same field with the new coloured polenta maize.

Polenta making

The new coloured R3246 polenta maize line was chosen and the seeds milled (Schnitzer, Grano 200, Germany) to obtain an ideal flour for polenta making (the source of the seeds material is described in "Material sampling" paragraph). The traditional recipe was used to obtain coloured and uncoloured polenta starting from the respective flours, but using distilled water to assure that no changes in the pigment molecules were due to the salts present in tap water. For the same reason no salt was added. Fifty grams of this flour were put in a beaker with 200 ml of distilled water (flour:water=1:4), heated up to 150°C and stirred. The boiling was held for 50 minutes, then the polenta was cooled down and weighed. The same procedure was also followed for the 'Scagliolo' control yellow variety.

Quantitative determination of Polenta Anthocyanins

The same procedure described in "Polenta making" paragraph was performed to quantify the anthocyanins content in the new coloured polenta. To assess the decrease in the polenta anthocyanins content caused by cooking, we compared the pigment amount in the fresh polenta to the expected one supposing no cooking effect on anthocyanins content. Hence we used the ratio polenta weight/flour weight to evaluate the "dilution factor" and to establish the expected polenta anthocyanin content to calculate the anthocyanins decrease due to cooking. The anthocyanin amount was calculated as cyanidin-3-glucoside equivalents for 100 g of dry flour as described above. The analyses were conducted on six independent preparations of polenta to calculate the confidence interval (C.I.) at 95% and the reduction of the amount of pigments due to cooking.

Antiradical ability

An equal amount of flour and polenta porridges of the coloured and uncoloured samples was shaken with acetone 70% (acetone:water 70:30 v/v) at room temperature. After 3 hours they were centrifuged for 10 minutes at 13000rpm and collected. The anthocyanin concentration was equalized between the red flour and the red polenta extracts, diluting an aliquot of them. The antioxidant ability was determine as the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging activity (Brand-Williams et al. 1995; Leong and Shui 2002; Cevallos-Casals and Cisneros-Zevallos 2003; Hu et al. 2004; Yang and Zhai 2010). Increasing aliquots of the yellow and red polenta extracts were added to a 0.12 m ethanolic DPPH solution to reach the final volume of 2.50 ml. The same solutions were also prepared for the red flour and red polenta equalized extracts, and for the yellow flour and polenta extracts as sampled. The samples were incubated for 3 hours at room temperature in the dark, then the absorbancies of the samples' decolorations were measured spectrophotometrically at 516 nm, comparing them with the ethanolic DPPH absorbance.

The percentage of DPPH was calculated as: % DPPH = (Ac−As) × 100 / Ac

where Ac is the absorbance of the control, and As is the absorbance of each increasing aliquot of the sample (Leong and Shui 2002; Hu et al. 2004; Yang and Zhai 2010). Then a curve was traced by interpolating the increasing aliquots of an extract with the corresponding DPPH scavenged percentage.

Extraction and qualitative determination of anthocyanins: TLC

To underline possible differences caused by the heating in polenta making, an aliquot of the flours and of the polenta of both the R3246 coloured line and the 'Scagliolo' control yellow variety were used to perform a TLC. The aliquots were boiled at 100°C with 2 ml of 2N HCl for 40 minutes, then ice chilled. After adding 1 ml of isoamyl alcohol, the upper phase was dried and suspended in EtOH 95% and HCl 1%. The coloured extracts were equalized according to the anthocyanin concentration and then loaded together with the main anthocyanidin standards on

a pre-coated plastic sheet (POLYGRAM CEL 300, MACHEREY-NAGEL) for Thin Layer Chromatography (TLC) using formic acid:HCl:water 5:2:3 as solvent. Developed plates were dried and pictures were taken with a digital camera (A430 Canon) using both white and UV illumination.

Panel Test/consumer test

Forty subjects were randomly recruited for a consumer test. These subjects were asked to taste blindly about 10g of the uncoloured and 10g of the coloured polenta, and to express their judgement according to a scale from 1, the worst, to 10, the best. The mean with the confidential interval at 95%, the median and the mode of the judgments relative to the two different kind of polenta were calculated.

Results

Development of coloured polenta maize lines through Marker Assisted Selection (MAS)

The yellow 'Scagliolo' polenta maize variety was used as the recurrent parent and crossed with a source of the dominant *B1* and *Pl1* anthocyanins regulatory genes (Fig. 1), that confer colour to the seed pericarp. The *B1b1 Pl1pl1* heterozygous individuals coming from the backcross cycles were selected and the genotype was confirmed by MAS. The *umc1014* SSR molecular marker, part of the *pl1* gene and the *umc1024* SSR molecular marker, associated with the *b1* gene was found to be polymorphic between the coloured and the colourless plants allowing us to select the *B1b1 Pl1pl1* heterozygotes in the backcrossing recurrent selection (Fig. SuppInfo1).

The selection was also based on quantification of the anthocyanin content in the kernels. These procedures allowed us to develop 4 new coloured polenta maize sub-lines, able to accumulate high levels of anthocyanins in the pericarp of the kernels (Fig. 2a).

Selection of the best coloured polenta line

The seeds' mean weight, their germinability and the anthocyanin content were analysed in the new coloured sub-lines and compared with the control line, yellow 'Scagliolo' maize, with the aim to find out the best coloured polenta maize sub-line (Table 1). The data showed mean seed weight values significantly lower in the coloured sub-lines compared to the yellow 'Scagliolo' variety: the coloured seeds mean weight ranging from 0.159g to 0.202g per line while the yellow seed mean weight was 0.254g (Table 1). The same tendency was shown for the germinability values, ranging from 52% to 84% in the coloured lines versus 92% for the 'Scagliolo' line, while these values appeared more variable (Table 1). The anthocyanin content data ranged from 55.78 mg/100g to 161.42 mg/100g in the coloured sub-lines (Table 1), while no anthocyanin was detected in the yellow 'Scagliolo' variety (Table 1). The comparison of these parameters among each sub-line allowed the selection of the best coloured one, which will be carried on in the breeding work, while the qualitative and nutritional analyses were performed on the R3246 sub-line thanks to its very abundant supply.

Nutritional properties

To test the nutritional properties of the new coloured line, seeds from a new coloured line and from the 'Scagliolo' yellow line, as the control, were used to prepare polenta. The R3246 seed supply was higher than that of the other coloured lines, so we used the seeds of this line to prepare polenta, according to the traditional Italian recipe. The same procedure was followed also for the uncoloured 'Scagliolo' variety. No obvious differences (e.g. consistence and firmness) were noticed during the cooking and in the final products, with the exception of the colour (Fig. 2b). Then we performed quantitative and qualitative analyses on the anthocyanins content in the flour before cooking and in the polenta after cooking to find out possible changes in the anthocyanins amount or in their chemical structure caused by the cooking procedure (the flour was stirred in boiling water for 50 minutes).

We first performed a pigment quantification to assess whether the cooking can cause the anthocyanins degradation. We have calculated the ratio between the weight of the flour and the weight of polenta coming from it for a better comparison of the anthocyanins amount in a dry (flour) vs a humid (polenta) compound. No anthocyanins were detected in the yellow 'Scagliolo' flour and polenta, as expected. These analyses found an anthocyanin amount of 57.71 mg/100g in the coloured flour and of 11.9 mg/100g in the corresponding polenta, adjusted to 44.77mg/100g, according to the flour weight used to make the polenta (Table 2).

Table 2 shows a statistically significant decrease of about 22% in the anthocyanin content of the polenta in comparison with the coloured flour.

We then tested the antioxidant ability of the anthocyanins present in the flours and in the polenta of the colourless and coloured samples.

The comparison of the curves of coloured and colourless polenta and coloured and colourless flour showed a higher radical scavenging ability in the anthocyanin rich one (Fig. 3a; Fig. S2). With regard to the coloured flour and polenta curves (Fig. 3b), in which the anthocyanin concentration was equalized, the results showed that the scavenging ability of the coloured polenta was a little lower than that of the coloured flour, despite the equalization. This small difference in the antioxidant ability is also evident in the comparison of the uncoloured flour and polenta curves (Fig. 3c).

Finally we performed TLC analysis to understand whether this quantitative reduction in the amount and in the scavenging ability affected some specific anthocyanin molecules (Fig. 4). The visible light picture of the plate (Fig. 5) showed first of all the standard spots of cyanidin and pelargonidin (Fig. 4, vis. lane 1 and 2). Then it shows the running patterns of the coloured flour and polenta equalized extracts (Fig. 4, vis. lane 3 and 4) and at the end the uncoloured flour and polenta ones (Fig.4, vis. lane 5 and 6). The visible spots of the coloured samples can be identified as the cyanidin and pelargonidin, according to the standards, and no differences seem to be present between these 2 samples (Fig. 4, vis. lane 3 and 4), indicating no changes in the molecule structure of the pigments

due to the cooking. No anthocyanin spots were detected in the uncoloured samples, as expected (Fig.4, vis. lane 5 and 6). In Figure 4 a UV light picture is also presented, showing an unidentified spot in each sample. Its amount seems to increase in the coloured polenta compared to the coloured flour (Fig. 4, UV lane 3 and 4). On the other hand this unidentified spot is much bigger in the uncoloured samples, between which no differences are perceived (Fig. 4, UV lane 5 and 6).

Panel Test/ consumer test

In a blind fold test on a consumer sample of 40 subjects the acceptability of the new coloured polenta was tested in comparison with the traditional yellow one. The acceptability scored a mean value of 5.4, a median value of 6 and a mode value of 5 for the yellow polenta (Table SuppInfo1), with scores of 5.5, 6 and 6 respectively for the coloured one (Table SuppInfo1). Statistical analysis showed no difference between the values assigned to coloured polenta in comparison to the yellow one (Table SuppInfo1).

Discussion

The aim of this work was to make the traditional Italian dish of polenta a healthier functional food by the use of an anthocyanin rich polenta maize line, developed in the University field of Landriano (PV). The development of this new coloured line needed to use both the classical breeding technique of the backcrossing scheme (Fig. 1) and the use of the more innovative molecular marker technique. In fact two SSR molecular markers, the umc1014 and umc1024 SSRs, assayed on the DNA of coloured and colourless polenta lines were found to be polymorphic (Fig. SuppInfo1), and thus were used for early selection of B1/b1 Pl1/pl1 heterozygous plants used in the recurrent selection. By this breeding scheme we obtained 4 sub-lines that accumulated high levels of anthocyanin pigments in the outer layer of the kernel, the pericarp (Fig. 2a). To select the polenta sub-line with the best features to become a functional food, we measure 3 parameters in each family in comparison to the yellow 'Scagliolo' control variety: the seed weight and the

germination ability, important from an agronomic point of view and the anthocyanin content (Table 1). The best pigment accumulator sub-line (R3077) displayed a level of anthocyanins of 161.42 mg/100g (Table 1), and also the highest seed weight among the new lines (0.202g), but the germination value was only 52% (Table 1). The R3076 sub line also showed a substantial amount of anthocyanin (about 147mg/100g), an higher germination (76%), but a low seed weight (0.159g) (Table 1), much lower than the 'Scagliolo' value of 0.254g (Table 1). The R3246 sub-line showed 0.168g for seed weight, only 60% for germination and 56 mg/100g of anthocyanins (Table 1). As shown in Table 1, a big variability regarding the anthocyanins content among the four sublines was observed ranging from 55.78 to 161.42 mg/100g of flour. Also a big intraline variability was noticed (data not shown): these data are explained by the behaviour of Pl1 gene. In fact Pl1 alleles can exist in quantitatively distinct regulatory states due to silencing phenomenon (Hollik et al. 2000; Pilu 2013) that modulate the anthocyanins accumulation. Hence the variability observed is mainly due to the silencing of the Pl1 gene whilst the environment has a secondary effect on this trait. Furthermore in every field season the anthocyanins content can change mainly because of the seasonal pattern of temperature.

The best combination seems to be presented by the R3075 sub-line with 0.198g of the seed weight, 84% of germination and 109.55mg/100g of anthocyanins content (Table 1). This line, shown in Figure 2a in comparison with the 'Scagliolo' variety, appeared to be a good candidate to become a functional food. During this sub-line's comparisons we noticed an inbreeding depression effect: the coloured polenta sub-lines always showed a statistically lower seed weight compared to the 'Scagliolo' variety, used as the control. The same effect was observed analysing the germination percentage. This inbreeding effect can be expected, due to the number of self pollination cycles performed. Moreover it must be considered that the 'Scagliolo' variety is a population, in fact it also showed a wider confidence interval (C.I.) when compared to the sub-lines. However, a lower value in the seed weight and in the germination percentage can compromise the commercial value of the new line. Actually we are planning to use this sub-line in a cross with

'Scagliolo', in order to obtain an F1- like generation; this could help to overcome this inbreeding depression problem. In this way a newly modified and traditional food can enter the dietary habits of lots of people, helping to increase the consumption of healthy compounds. We then analysed some quality traits.

The R3246 sub-line seed supply was much more abundant compared to the others, so it was used to perform these analyses. First of all it was necessary to quantify the anthocyanin content in the polenta, and to analyse whether the cooking of the flour changed the anthocyanin molecules. No particular differences were noticed during this procedure, apart from, of course, the colour (Fig. 2b). In the 'Scagliolo' polenta no anthocyanins were detected, while the pigment content in the coloured one was about 12 mg/100g of polenta food (Table 2). This amount seems to be low, when compared it with the anthocyanin content in berries (i.e. blueberries anthocyanins content: from 25 to 495 mg/100g (Mazza and Miniati 1993). However the United States daily intake is estimated to be 12.5 mg of anthocyanins per capita (Wu et al. 2006), while a polenta serving, generally made with 125g of flour, could offer from 56mg (in the case of this sub-line) to around 99mg (in the case of the best sub-line) per capita. Considering these results the coloured polenta can be considered a new functional food allowing an increased anthocyanins intake.

The analyses showed a decrease of about 22% in the anthocyanins content of polenta when compared to the flour, probably due to the cooking process. The decrease is statistically significant, but is not considered high, especially when compared to that due to microwave treatment on purple popcorn (Lago et al. 2012). However this loss was not unexpected because it is known that anthocyanin content and stability in the final products are affected by both temperature and food processing (Giusti and Wrolstad 2003; Jackman and Smith 1996). For example heating produced a decrease of about 50% in the anthocyanin content of elderberry (Sadilova et al. 2006), in raspberry purees (Ochoa et al. 1999) and also in the total phenols of baked muffins enriched with red raspberry juice (Rosales-Soto et al. 2012).

Moreover a lixiviation phenomenon often occurs during the boiling (Bunea et al. 2008): the hydrophilic phenols enter the water forming protein complexes (Barroga et al. 1985; Rocha-Guzman et al. 2007) that leads to about 50% of phenol loss (Bunea et al. 2008; Jiménez-Monreal et al. 2009).

Also Salinas-Moreno et al. (2003) found that anthocyanins amount was significantly reduced by cooking maize in water (Salinas-Moreno et al. 2003; Jones 2007). However we must consider that in the polenta preparation water remains an essential component of the food preparation; this could be the explanation for the limited loss of anthocyanin content that we detected in coloured polenta. We also checked the antioxidant capability of the coloured polenta to find out whether the decrease of these pigments was coupled with a loss in the scavenging ability, and whether the cooking treatment was able to change the anthocyanins' molecules' structures.

First of all the DPPH scavenging ability was assayed by comparing the uncoloured and coloured polenta: the red one showed a higher antioxidant power in comparison with the yellow one (Fig. 3a). This result was expected: by now the antioxidant capacity of the anthocyanins and of the flavonoids in general is renowned, given their ability to donate phenolic hydrogen atoms (Chen et al. 1996; Urquiaga and Leighton 2000; Nijveldt et al. 2001). This is an encouraging result for our effort to develop and to characterize a new functional food although as shown in Fig.S3 the relationship between pigment content and antioxidant capacity seems not linear as previously reported by Rodriguez et al., 2013, obviously it could depend also on the procedure used. For this reason deeper and more accurate analyses will be necessary to clarify this issue. Moreover, to better understand whether the cooking procedure causing the decrease of anthocyanin content was also able to change the pigments' structure and their antioxidant capability, the scavenging ability was compared between the coloured extracts of the flour and polenta, diluted to equalize the anthocyanins amount. The results showed that the red flour keeps a little higher power, despite the equalization (Fig. 3b).

This could be due to some antioxidant substances other than the anthocyanins, present in maize, that contribute to the overall scavenging ability but that cannot

withstand the heat of cooking. For example ascorbic acid in several vegetables (Yamaguchi et al. 2001) and carotenoids in spinach (Bunea et al. 2008) are negatively affected by boiling. The analysis of the DPPH activity of the uncoloured flour and polenta seems to strengthen this hypothesis: a decrease in the scavenging ability after cooking is also shown in corn with no anthocyanins (Fig. 3c), proving the presence of uncoloured antioxidant substances. In fact, for what concerns phenolic compounds, it is known that phenolic acids and flavonoids are commonly present in whole maize kernel (Žilić S et al., 2012). In particular ferulic acid seems to be the predominant phenolic compound (Fry, 1986; Bily et al., 2003; Tokuji et al., 2009), while p-coumaric acid (pCA) is the second most frequent phenolic acid in maize (Bily et al., 2003). Furthermore, we performed a TLC analysis on the anthocyanin extract made from coloured flour and polenta, to better dissect the possible effect of the heat of cooking on the pigments' structure. In the visible part of the plate (Fig. 4) the coloured flour spots resulting from the flour extracts are cyanidin and pelargonidin, according to the standards, as expected. The same situation is shown also for the polenta extracts. In the end the results showed no differences in the anthocyanins ratio (Fig. 4), suggesting that the cooking causes a decrease in the anthocyanin molecules, but not a change in their structure or in their relative amount, as already found in purple popcorn making (Lago et al. 2012). The last 2 samples loaded on the plate were the 'Scagliolo' flour and polenta extracts (Fig. 4): no anthocyanin spots were present in the visible light, as expected in yellow varieties. Interestingly the UV picture of the TLC pointed out an unidentified spot in each sample, that seemed to change. In fact while in the uncoloured samples the cooking does not change this spot (Fig. 4, UV, lane 5 and lane 6), in the coloured samples the heating seems to increase its amount (Fig. 4, UV, lane 3 and lane 4). This finding could be in accordance with the result obtained by Hiemori et al. (2009) showing that cyanidin-3-glucoside in California black rice is degraded during cooking into colorless protocatechuic acid able to absorb UV radiation. We can speculate that the increased unidentified spot after cooking may be due to a degradation of the anthocyanins, probably into

protocatechuic acid. To confirm this hypothesis, however, more intensive chemical analyses are needed.

Finally, it can be said that despite the decrease due to cooking, the coloured polenta retained an appreciable amount of healthy compound and a good relative level of antioxidant activity, so as to be considered a new functional food. Nevertheless another important parameter to take into account is the consumers' rating of acceptability, fundamental when a new product is going to enter the market. The judgment of the 40 blinded subjects scored mean values of 5.45±0.618 for the yellow 'Scagliolo' polenta and 5.57±0.572 for the coloured one (Table SuppInfo1) and no statistical differences emerged (Table SuppInfo1). These degrees of appreciation may seem as if they are not very high on a scale starting from 1, the lowest, to 10, the highest. However we have to consider that the subjects were asked to pass judgment about a food prepared without salt or dressing, while it is traditionally eaten with sauces. Thus the instructive data remains the lack of difference between the flavour and texture acceptability rating between the 2 types of polenta.

The results obtained show that the newly developed coloured polenta exhibited a good antioxidant capacity compared to the colourless control, even though a degradation of about 22 % in the anthocyanin content relative to the flour, was caused by cooking. Furthermore the taste perception by consumers revealed no differences between coloured and uncoloured polenta. These results suggested that the coloured polenta could be chosen by the consumers in preference to the uncoloured one, because of its healthier aspect. Taken into account all the results presented the development of the new polenta variety, rich in the anthocyanin pigments, would appear to be a good tool to increase the antioxidant power in the diet.

Acknowledgements

We wish to thank Dr. Davide Reginelli for his hard work in the field and the two anonymous reviewers for their comments and precious suggestions.

Figures and Tables

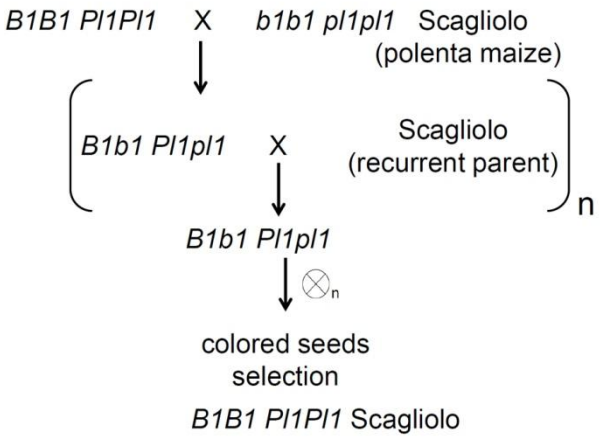


Figure 1. Recurrent Selection Scheme: after the first cross between the line B1Pl1, source of the regulatory biosynthetic genes and the traditional 'Scagliolo' variety, the ears heterozygous for the B- Pl- genes and with the highest anthocyanin content were selected for backcrossing. The 4 resulting sub-lines were further selected for the highest levels of anthocyanins and underwent some cycles of self-pollination.

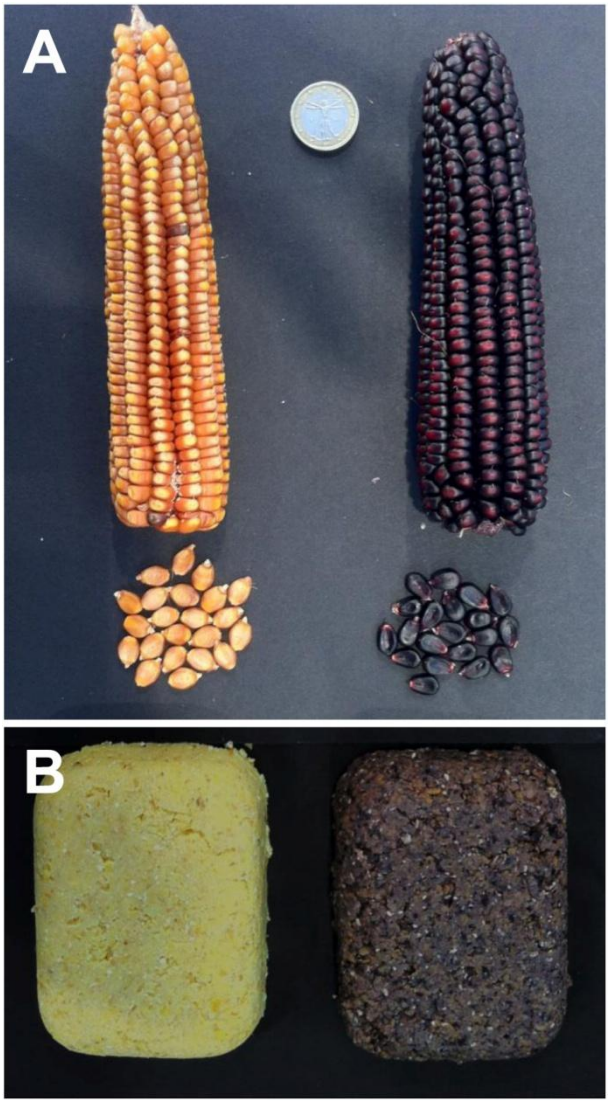


Figure 2. Phenotype of ear and kernels (a), and polenta (b) of the new coloured polenta line (right) and of the traditional colourless 'Scagliolo' one (left).

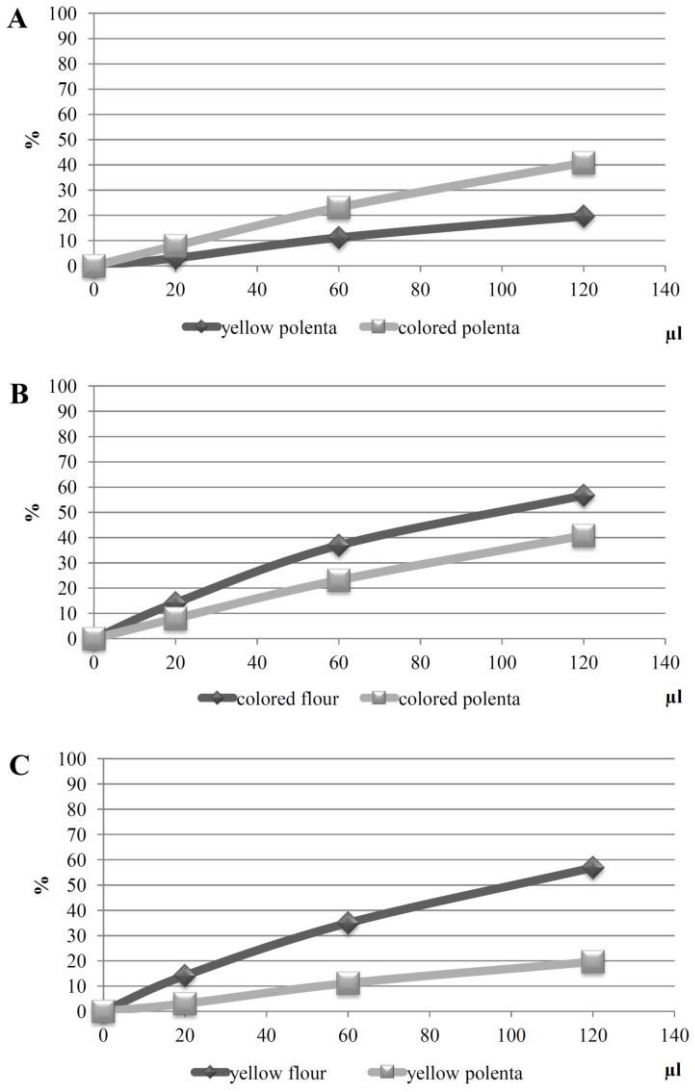


Figure 3. Comparison of the antioxidant ability in the DPPH radical scavenging test of coloured and uncoloured polenta (a), between coloured flour and polenta (b) and between uncoloured flour and polenta (c).

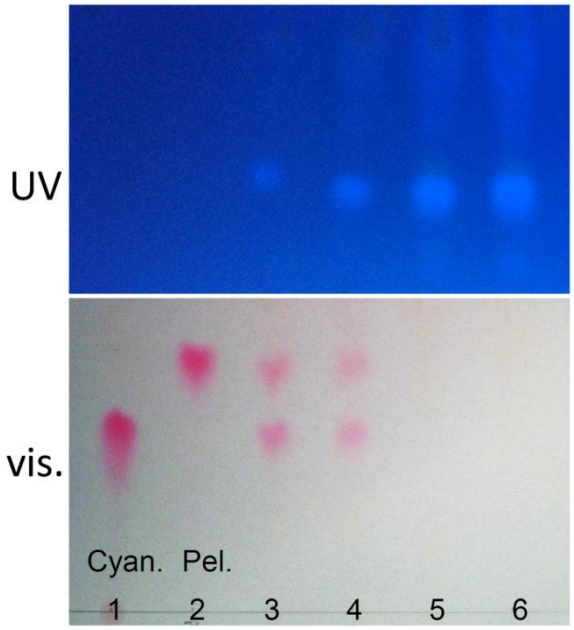


Figure 4. Pictures of the TLC plate taken under visible or UV light. The spots represented are cyanidin (1) and pelargonidin (2) standards, the anthocyanin extracts from the coloured flour (3) and polenta (4) and the extracts from the uncoloured 'Scagliolo' flour (5) and polenta (6)

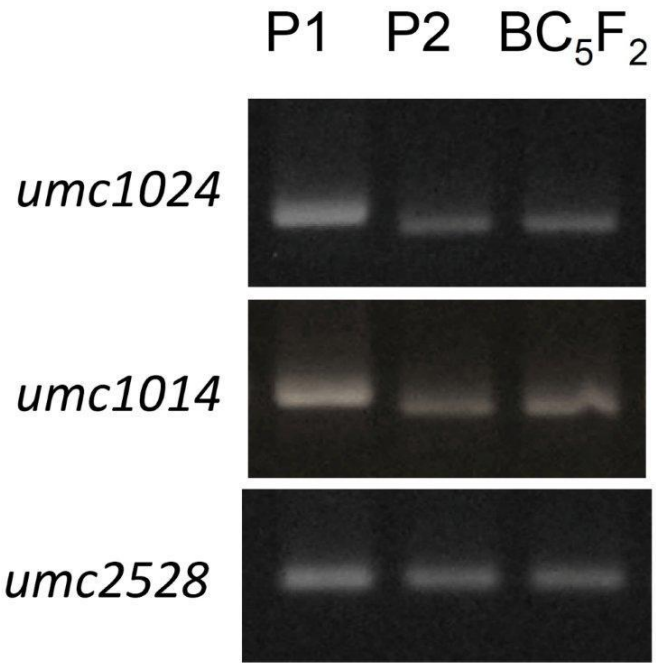


Figure S1. Marker-assisted selection: two SSRs were polymorphic between the coloured and the colourless polenta lines. The umc1014 is the SSR part of the Pl1 gene and the umc1024 SSR is associated with the B1 gene. The umc2528 SSR, associated with the R gene not involved in the selection, and did not show polymorphism between the two polenta varieties. The heterozygous individuals were detected and selected to carry on the breeding selection.P1 parent 1; P2 parent 2; BC back crossing.

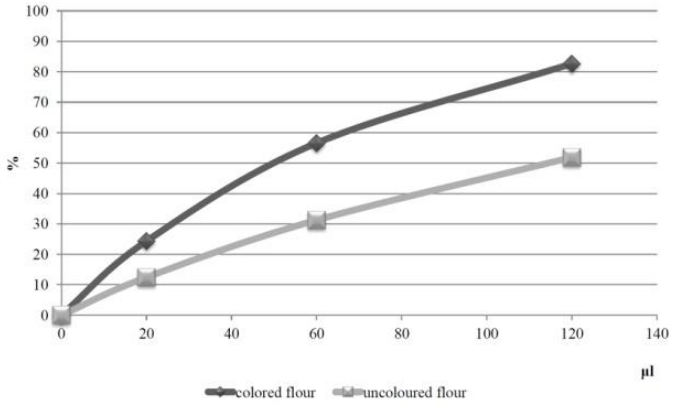


Figure S2. Comparison of the antioxidant ability in the DPPH radical scavenging test of coloured and uncoloured flour.

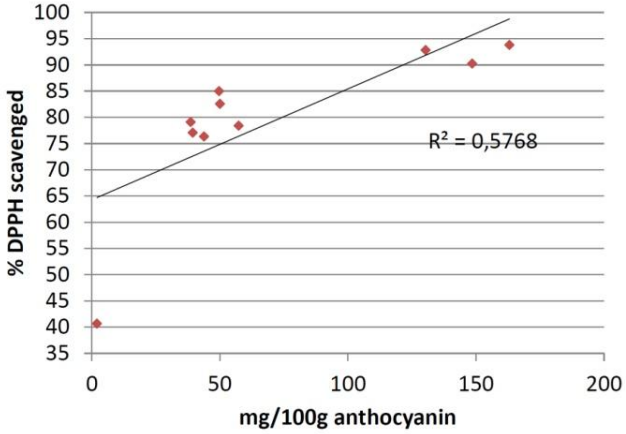


Figure S3. Correlation analysis between the anthocyanins content and the scavenging ability.

Table 1: Values of the traits measured in the colourless 'Scagliolo' variety of maize and in the coloured sub-lines. Confidence Intervals at 95% are shown.

	Mean Weight seed (g)	**Germination (%)**	**Anthocyanins (mg/100g)**
'Scagliolo'	0.254±0.040	92±10.85	N.D.[a]
R3075	0.198±0.004	84±14.67	109.55±24.01
R3076	0.159±0.002	76±17.09	146.68±28.54
R3077	0.202±0.007	52±19.99	161.42±26.85
R3246	0.168±0.005	60±19.60	55.78±16.13

Table 2: Anthocyanin amounts quantified in the flour and in the polenta of the chosen coloured sub-line and in the colourless 'Scagliolo' variety. To calculate the decrease of the anthocyanins content caused by cooking, we compared the pigment amount in the fresh polenta to the expected one (considering the anthocyanins amount in the flour and the weigh change due to the polenta preparation). Confidence Intervals at 95% are shown.

Anthocyanins	**Flour (mg/100g)**	**Polenta (mg/100g)**		**Decrease**
		obtained	**expected**	
Uncoloured	N.D.[a]	N.D.[a]	-	-
Coloured	57.71 ± 7.1	11.90± 1.15	15.34 ± 2.5	22.4 %

[a]Not Detected

Table S1. Mean, median and mode of the degree of preference for the colourless 'Scagliolo' polenta in comparison with the new coloured one in blindfolded trials. Confidence Intervals at 95% are shown.

	Colourless	**Coloured**
Mean	5.45 ± 0.618	5.575 ± 0.572
Median	6	6
Mode	5	6

References

Barroga, F.C., A. C. Laurena, and E. M. T. Mendoza, 1985: Polyphenols in Mung bean (Vigna radiate, L. Wilczek): determination and removal. J. Agric. Food Chem. 33, 1006–1009.

Berardo, N., O. V. Brenna, A. Amato, P. Valoti, V. Pisacane, and M. Motto, 2004: Carotenoids concentration among maize genotypes measured by near infrared reflectance spectroscopy (NIRS). Innov. Food Sci. Emerg. 5, 393– 398.

Bily A.C., Reid L.M., Taylor J.H., Johnston D., Malouin C., Burt A.,J., Bakan B., Regnault-Roger C., Pauls K.P., Arnason J.T., and B.J.R. Philogène, 2003: Dehydrodimers of Ferulic Acid in Maize Grain Pericarp and Aleurone: Resistance Factors to Fusarium graminearum. Phytopathology 93, 712-719

Bodeau, J. P., and V. Walbot, 1992: Regulated transcription of the maize Bronze-2 promoter in electroporated protoplasts requires the C1 and R gene products. Mol. Gen. Genet. 233, 379-387.

Brand-Williams, W., M. E. Cuvelier, and C. Berset, 1995: Use of a free radical method to evaluate antioxidant activity. Lebensm. Wiss. Technol. 28, 25-30.

Brandolini, A, and A. Brandolini, 2009: Maize introduction, evolution and diffusion in Italy. Maydica 54, 233-242.

Bunea, A., M. Andjelkovic, C. Socaciu, O. Bobis, M. Neacsu, R. Verhé, and Van Camp J., 2008: Total and individual carotenoids and phenolic acids content in fresh, refrigerated and processed spinach (Spinacia oleracea L.). Food Chem. 108, 649–656.

Castilla, P., A. Dávalos, J.L. Teruel, F. Cerrato, M. Fernández-Lucas, J. L. Merino, C. C. Sánchez-Martín, J. Ortuño, and M. A. Lasunción., 2008: Comparative effects of dietary supplementation with red grape juice and vitamin E on production of superoxide by circulating neutrophil NADPH oxidase in hemodialysis patients. Am. J. Clin. Nutr. 87, 1053–1061.

Cevallos-Casals, B. A., and L. Cisneros-Zevallos, 2003: Stoichiometric and kinetic studies of phenolic antioxidants from Andean purple corn and red-fleshed sweetpotato. J. Agr. Food Chem. 51, 3313-3319.

Chandler, V. L., J. P. Radicella, T. P. Robbins, J. Chen, and D. Turks, 1989: Two regulatory genes of the maize anthocyanin pathway are homologous: isolation of B utilizing R genomic sequences. Plant Cell 1, 1175–1183.

Chen, Z. Y., P. T. Chan, K. Y. Ho, K. P. Fung, and J. Wang, 1996: Antioxidant activity of natural flavonoids is governed by number and location of their aromatic hydroxyl groups. Chem. Phys. Lipids 79, 157-163.

DeFuria, J., G. Bennett, K. J. Strissel, J. W. 2nd. Perfield, et al., 2009: Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae. J. Nutr. 139, 1510–1516.

de Pascual-Teresa, S., C. Santos-Buelga, J. C. Rivas-Gonzalo, 2002: LC-MS analysis of anthocyanins from purple corn cob. J. Sci. Food Agr. 82, 1003-1006.

Dellaporta, S. L., J. Wood, and J.B. Hicks, 1983: A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1, 19-21.

Dickerson, G. W., Extension Horticulture Specialist, Specialty Corns. Reprinted February 2003

Dooner, H. K., T. P. Robbins, and R. A. Jorgensen, 1991: Genetic and developmental control of anthocyanin biosynthesis. Annu. Rev. Genet. 25, 173–199.

Escribano-Bailón, M. T., C Santos-Buelga, J. C. Rivas-Gonzalo, 2004: Anthocyanins in cereals. J. Chromatogr. A 1054, 129–141.

Food and Agriculture Organization of the United Nations, 2009: Crops Production. http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567. Accessed 20 April 2011

Fry S.C. 1986: Cross-Linking of matrix polymers in the growing cell walls of angiosperms. Ann Rev Plant Physiol 37, 165-186

Gaut, B. S., 2001: Patterns of chromosomal duplication in maize and their implications for comparative maps of the grasses. Genome Res. 11, 55-66.

Giusti, M. M., and R. E. Wrolstad, 2003: Acylated anthocyanins from edible sources and their applications in food systems. Biochem. Eng. J 14, 217-225.

Goyarzu, P., D. H. Malin, F. C. Lau, G. Taglialatela, et al., 2004: Blueberry supplemented diet: effects on object recognition memory and nuclear factor-kappa B levels in aged rats. Nutr. Neurosci. 7, 75–83.

Hiemori, M., E. Koh, and A. E. Mitchell, 2009: Influence of cooking on anthocyanins in Black Rice (Oryza sativa L. japonica var. SBR). J. Agr. Food Chem. 57, 1908–1914.

Hollick, JB., Patterson G.I., Asmundsson I.M. and Chandler V.L. 2000: Paramutation alters regulatory control of the maize pl locus. Genetics 154, 1827–1838.

Hu, F. L., R. L. Lu, B. Huang, L. Ming, 2004: Free radical scavenging activity of extracts prepared from fresh leaves of selected Chinese medicinal plants. Fitoterapia 75, 14−23.

Iwasaki-Kurashige, K., R. Y. Loyaga-Rendon, H. Matsumoto, et al., 2006: Possible mediators involved in decreasing peripheral vascular resistance with blackcurrant concentrate (BC) in hind-limb perfusion model of the rat. Vasc. Pharmacol. 44, 215–223.

Jackman, R. L., and J. L. Smith, 1996: Anthocyanin and betalains. In: G. A. F. Hendry and J. D. Houghton (ed.), Natural food colorants, 249-309. Blackie Academic and Professional, Glasgow.

Jiménez-Monreal, A. M., L García-Diz, M. Martínez-Tomé, M. Mariscal, and M. A. Murcia, 2009: Influence of cooking methods on antioxidant activity of vegetables. J. Food Sci. 74, H97-H103.

Jones, R. B., 2007: Effects of postharvest handling conditions and cooking on anthocyanin, lycopene, and glucosinolate content and bioavailability in fruits and vegetables. New Zeal. J. Crop Hort. 35, 219-227.

Lago, C., M. Landoni, E. Cassani, E. Doria, E. Nielsen, and R. Pilu, 2012: Study and characterization of a novel functional food: purple popcorn. Mol Breeding. 31, 575–585

Lau, F. C., D. F. Bielinski, and J. A. Joseph, 2007: Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated BV2 microglia. J. Neurosci. Res. 85, 1010–1017.

Leong, L. P., and G. Shui, 2002: An investigation of antioxidant capacity of fruits in Singapore markets. Food Chem. 76, 69-75.

Matsumoto, H., K. E. Kamm, J. T. Stull, and H. Azuma, 2005: Delphinidin-3-rutinoside relaxes the bovine ciliary smooth muscle trough activation of ETB receptor and NO/cGMP pathway. Exp. Eye Res. 80, 313–322.

Mazza, G., and E. Miniati, 1993: Anthocyanins in fruits, vegetables, and grains. CRC Press, Boca Raton, FL

Nakatani, N., H. Fukuda, H. Fuwa, 1979: Major anthocyanins of Bolivian purple corn (Zea mays L.). Agri. Biol. Chem. 43, 389-391.

Nijveldt, R. J., E. van Nood, D. E. van Hoorn, P. G. Boelens, K. van Norren, and P. A. van Leeuwen, 2001: Flavonoids: a review of probable mechanisms of action and potential applications. Am. J. Clin. Nutr. 74, 418–25.

Ochoa, M. R., A. G. Kesseler, M. B. Vullioud, and J. E. Lozano, 1999: Physical and chemical characteristics of raspberry pulp: storage effect on composition and colour. Lebensm. Wiss. Technol. 32, 149-153.

Panzeri, D., V. Cesari, I. Toschi, and R. Pilu, 2011: Seed calorific value in different maize genotypes. Energy Sourc. Part A Recov. Utiliz. Environ. Eff. 33, 1700-1705.

Peng, C.H., L. K. Liu, C. M. Chuang, et al., 2011: Mulberry water extracts possess an anti-obesity effect and ability to inhibit hepatic lipogenesis and promote lipolysis. J. Agr. Food Chem. 59, 2663–2671.

Pilu, R., P. Piazza, K. Petroni, A. Ronchi, C. Martin, and C. Tonelli, 2003: pl-bol3, a complex allele of the anthocyanin regulatory pl1 locus that arose in a naturally occurring maize population. Plant J. 36, 510–521.

Pilu, R. 2011: Paramutation: just a curiosity or fine tuning of gene expression in the next generation? Curr Genomics 12, 298–306

Prior, R. L., X. Wu, L. Gu, et al., 2008: Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity. J. Agr. Food Chem. 56, 647–653.

Rissanen, T. H., S. Voutilainen, J. K. Virtanen, et al., 2003: Low intake of fruits, berries and vegetables is associated with excess mortality in men: the Kuopio Ischemic Heart Disease Risk Factor (KIHD) Study. J. Nutr. 133, 199–204.

Rocha-Guzmán, N. E., R. F. González-Laredo, F. J. Ibarra-Pérez, C. A. Nava-Berúmen, and J. A. Gallegos-Infante, 2007: Effect of pressure-cooking on the antioxidant of extracts from three common bean (Phaseolus vulgaris L.) cultivars. Food Chem. 100, 31–5.

Rodriguez-Amaya, D. B., M. Kimura, H. T. Godoy, and J. Amaya-Farfan, 2008: Updated Brazilian database on food carotenoids: Factors affecting carotenoid composition. J. Food Compos. Anal. 21, 445– 463.

Vıctor, M. R., Soengas, P., Landa A., , Ordas A., Revilla P., 2013: Effects of selection for color intensity on antioxidant capacity in maize (Zea mays L.) Euphytica 193, 339–345

Rosales-Soto, M. U., J. R. Powers, and J. R. Alldredge, 2012: Effect of mixing time, freeze-drying and baking on phenolics, anthocyanins and antioxidant capacity of raspberry juice during processing of muffins. J. Sci. Food Agr. 92, 1511-1518.

Sadilova, E., F. C. Stintzing, R. Carle, 2006: Thermal degradation of acylated and non acylated anthocyanins. J. Food Sci. 71, C504-C512.

Salinas-Moreno Y., F. Martinos-Bustos, M. Soto-Hernandez, R. Ortega-Paczka, and J. L. Arellano-Vazquez, 2003: Effect of alkaline cooking process on anthocyanins in pigmented maize grain. Agrosciencia 37, 617-628.

Sebrell, W. H., 1981: History of pellagra. Federation Proceedings 40, 1520-1522.

Seymour, E. M., S. K. Lewis, D. E. Urcuyo-Llanes, et al., 2009: Regular tart cherry intake alters abdominal adiposity, adipose gene transcription, and inflammation in obesity-prone rats fed a high fat diet. J. Med. Food 12, 935–942.

Shukitt-Hale, B., F. C. Lau, A. N. Carey, et al., 2008: Blueberry polyphenols attenuate kainic acid-induced decrements in cognition and alter inflammatory gene expression in rat hippocampus. Nutr. Neurosci. 11, 172–182.

Titta, L., M. Trinei, M. Stendardo, et al., 2010: Blood orange juice inhibits fat accumulation in mice. Int. J. Obesity 34, 578–588.

Toufektsian, M. C., M. de Lorgeril, N. Nagy, et al., 2008: Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. J. Nutr. 138, 747–752.

Tsuda, T., 2008: Regulation of adipocyte function by anthocyanins; possibility of preventing the metabolic syndrome. J. Agr. Food Chem. 56, 642–646.

Tsuda, T., 2012: Dietary anthocyanin-rich plants: biochemical basis and recent progress in health benefits studies. Mol. Nutr. Food Res. 56, 159-170.

Urquiaga, I., and F. Leighton, 2000: Plant polyphenol antioxidants and oxidative stress. Biol. Res. 33, 55–64.

Venturelli, M. B., B. Purin, and M. Pirola, 1990: Maize for the production of polenta flour. Informatore Agrario 46, 70–71, 128–129.

Virgili, F., and M. Marino, 2008: Regulation of cellular signals from nutritional molecules: A specific role for phytochemicals, beyond antioxidant activity. Free Radical Bio. Med. 45, 1205–1216.

WHO Noncommunicable diseases country profiles 2011 ISBN 978 92 4 150228 3 http://www.who.int/topics/chronic_diseases/en/. Accessed march 2012

Wu, X., G. R. Beecher, J. M. Holden, D. B. Haytowitz, S. E. Gebhardt, R. L. Prior, 2006: Concentration of anthocyanins in common foods in the United States and estimation of normal consumption. J. Agr. Food Chem. 54, 4069–4075.

Yamaguchi, T., T. Mizobuchi, R. Kajikawa, H. Kawashima, F. Miyabe, J. Terao, H. Takamura, and T. Matoba, 2001: Radical-scavenging activity of vegetables and the effect of cooking on their activity. Food Sci. Technol. Res. 7, 250–257.

Yang, Z., and W. Zhai, 2010: Identification and antioxidant activity of anthocyanins extracted from the seed and cob of purple corn (Zea mays L.). Innov. Food Sci. Emerg. 11, 169–176.

Zeppa, G., M. Bertolino, and L. Rolle, 2012: Quantitative descriptive analysis of Italian polenta produced with different corn cultivars. J. Sci. Food Agr. 92, 412–417.

Žilić S., Serpen A., Akıllıoğlu G., Gökmen V. and J. Vančetovic (2012) Phenolic Compounds, Carotenoids, Anthocyanins, and Antioxidant Capacity of Colored Maize (Zea mays L.) Kernels. J Agric Food Chem 60,1224−123.

Development and characterization of a coloured sweet corn line as a new functional food

Chiara Lago[1], Michela Landoni[2], Elena Cassani[1], Samuel Attanasiu[1], Roberto Pilu[1].

[1] Dipartimento di Scienze Agrarie e Ambientali - Produzione, Territorio, Agroenergia, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy

[2] Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

This is a pre-copy-edyting, author-produced PDF of an article submitted for publication in PLANT BIOTHENOLOGY REPORTS *following peer review.*

Lago C, Landoni M, Cassani E, Attanasiu S, Pilu R. Development and characterization of a coloured sweet corn line as a new functional food.

The defenitive piblisher-authenticated version will be avalaible online at:

http://link.springer.com/journal/volumesAndIssues/11816

Abstract

Sweet corn (*Zea mays saccharata* Sturt.) is a maize variety grown for the fresh, frozen and canned markets. The standard sugary (*su*) sweet corn possesses a flavour and a texture traditionally appreciated. Its kernels are characterized by the presence of high free sugars and some antioxidant substances, in particular ferulic acid, suggested to be beneficial for cancer prevention. For this reason an interesting challenge for breeders is the development of sweet corn genotypes with naturally high antioxidant levels, starting from flavonoids. In fact other important sources of antioxidants in maize are anthocyanins, considered as nutraceuticals because they have been proven to lower the risk of many chronic diseases. In this paper we report the development of a new coloured sugary line and the uncoloured commercial control and the results of some analyses concerning their flavonoid content before and after 2 different cooking treatments are discussed. Attention was mainly focused on the anthocyanins, the molecules suggested as being responsible for the nutraceutical properties of the new coloured sugary line. The results show that the presence of the anthocyanins also pushes up the flavonol and the phenolic acid amounts and gives the new coloured sugary line a higher scavenging power compared to the uncoloured control. The mild cooking seems not to significantly change the metabolites analyzed in the coloured kernels, while the stronger treatment seems to drastically decrease the amounts of pigments, without changing the structure of the leftover molecules. All these findings suggest that the new coloured sugary line can be considered a new functional food, able to introduce healthy compounds into the diet of many people.

Key words: Maize, *Sugary*, Anthocyanins, *Purple plant 1*, *Booster 1,* Functional food

Introduction

According to the data for the years 2012-2013, the corn global yield overtook 850 million metric tonnes, so that maize can now be considered as the most produced cereal in the world (USDA data). Corn is characterized by a high versatility: it is used for food, forage and for industrial purposes. In USA the amount of corn used as food is about 1.4 billion bushels (35.56 million metric tonnes), to produce high-fructose corn syrup, starch, corn oil and various other food products. (Brester 2012. http://www.agmrc.org/commodities__products/grains__oilseeds/corn_grain/). Among the different varieties of maize used for different purposes an important one is sweet corn. Sweet corn (*Zea mays saccharata* Sturt.) is a corn type grown for fresh, frozen and canned markets (Bülent Coşkun et al. 2006). In USA the fresh market accounts for nearly 70% of the total production of the sweet corn crop, and it is the second largest processing crop, surpassed only by tomatoes (Hansen R., content specialist, AgMRC, Iowa State University, Sweet corn profile http://www.agmrc.org/commodities__products/grains__oilseeds/corn_grain/sweet-corn-profile/; Haynes et al., Sweet Corn, Iowa State University Horticulture Guide). It differs from starchy field corn by a single recessive naturally-occurring genetic mutation causing a higher sugar content in the kernels. As a consequence sweet corn is harvested during the milk stage, before physiological maturation, approximately 15 to 23 days after the silks emerge, 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 mutations resulting in the three most widely diffused genetic varieties of sweet corn: sugary (*su*), sugarenhanced (*se*), and shrunken-2 (*sh2*): they vary in sweetness, shelf life and cold soil vigour. The most diffused and ancient sweet corn variety is the sugary. This variety has a harvest, storage and shelf life slightly shorter than the others, the sugar content is not so high compared to *se* and *sh2*, but it is characterized by a flavour and a texture traditionally appreciated by consumers.

The sweet corn kernels are characterized by a high starch and sugar content, important energy sources, by cellulose and β-glucan which are important dietary fibre for the enteric flora (Topping and Clifton 2001; Tokuji et al. 2009) and by the presence of zinc, an essential mineral to assure the functioning of many enzymes and transcription factors

(Haase et al. 2008; Tokuji et al. 2009). Some antioxidant substances can also be found in sweet corn kernels, such as the β-carotene and the lutein carotenoids (Kurilich and Juvik 1999; Tokuji et al. 2009) and above all the phenolic compound ferulic acid (Balasubashini et al. 2003; Tokuji et al. 2009). This molecule seems to be very important for health, in fact Tokuji and colleagues collected data indicating that this compound found in dietary sweet corn can be beneficial for cancer prevention (Tokuji et al. 2009). The antioxidant power seems to be the mechanism through which the molecules carry out their preventive function against human chronic diseases (Virgili and Marino 2008), and cancer. Therefore vegetable foods containing high levels of antioxidant compounds are now entering the human diet as essential constituents, endowed with the added value of the functional food. So developing sweet corn genotypes with naturally high antioxidant level could be an interesting challenge for breeders. Important sources of antioxidants in maize are the anthocyanins. Anthocyanins are a class of flavonoids: they are water-soluble glycosides of simple or acylated polyhydroxy and polymethoxy derivates of flavylium salts and they are responsible for the red, purple, and blue colours of many fruits, vegetables, and cereal kernels (Giusti and Wrolstad 2003; Zilic et al. 2012). They are very important for human health because they have been proven in animal system to reduce the risk of death from heart disease (Rissanen et al. 2003; Tsuda 2012), to be able to lower LDL-cholesterol levels (Castilla et al. 2008; Tsuda 2012) and to fight obesity (Seymour et al. 2009; Titta et al. 2010; Peng et al. 2011; Tsuda 2012) and diabetes (Tsuda 2008; Prior et al. 2008; DeFuria et al. 2009; Tsuda 2012), to improve visual function (Matsumoto et al. 2005; Iwasaki-Kurashige et al. 2006) and to prevent neurodegenerative diseases (Goyarzu et al. 2004; Lau et al. 2007; Shukitt-Hale et al. 2008; Tsuda 2012).

Corn (*Zea mays* L.) contains around 20 structural and regulatory genes that compose the anthocyanin biosynthetic pathway (Chandler et al. 1989; Dooner et al. 1991; Pilu et al. 2003). Native maize varieties cultivated in South America were coloured (Zilic et al. 2012). The regulatory genes concerned belong to two different multigene families: the class of bHLH transcription factors among which are the *r1/b1* genes, and the class of MYB transcription factors, among which are the *c1/pl1/p1* genes (Chandler et al. 1989; Dooner et al. 1991; Pilu et al. 2003). A member of each family must be present and active in the dominant form to activate anthocyanin structural gene expression. According to the combination of these alleles, the pigments will be synthesized in different plant tissues,

for example the *B/Pl* genes combination confers purple colour to the pericarp (Chandler et al.1989; Bodeau and Walbot 1992; Gaut 2001; Pilu et al. 2003).

In this paper we describe how a new coloured sugary line has been developed and, together with an uncoloured control, was subjected to three different food processing treatments: raw, steam cooked and autoclaved. Some analyses concerning the quantitative and qualitative characterization of the main flavonoid molecules in the uncoloured and coloured samples are presented and the results obtained after the different cooking treatments are discussed. Attention has been centred on the anthocyanins, the molecules that are supposed to be responsible for the nutraceutical properties for the new coloured sugary line, which is therefore proposed as a new functional food.

Materials and methods

Plant material

A backcrossing breeding scheme was used to develop a sugary maize line rich in anthocyanins, in the experimental field of the University of Milan located in Landriano (PV, Italy). The source of the anthocyanin biosynthesis regulatory genes was a tropical maize line carrying the homozygous form of the *Booster1* (*B1*) and *Purple Plant1* (*Pl1*) genes, that determine the pigmentation in the pericarp and in the plant. This line was crossed with a commercial yellow sugary line, used as the recurrent parent for 5 cycles of backcrossing. Then 3 cycles of self pollination were performed, selecting in each cycle, the plants with the highest content of anthocyanins by Marker Assisted Selection (MAS).

Molecular Marker assay

Two SSR molecular markers were used to select the coloured sugary plants: the *nc009* SSR molecular marker (5'CGAAAGTCGATCGAGAGACC3'/ 5'CCTCTCTTCACCCCTTCCTT3'), that is part of the *pl1* gene located on chromosome 6 and the *bnlg1064* SSR molecular marker (5'CTGGTCCGAGATGATGGC3'/5'TCCATTTCTGCATCTGCAAC3') located next to the *b1* gene on the short arm of chromosome 2 (http://www.maizeg db.org/ssr.php). After the DNA extraction from the leaves of parental (P1 and P2) and progenies' plants (Dellaporta et al. 1983), the Polymerase Chain Reactions (PCR) and gel running were

performed as described in the SSR Methods Manual by MaizeGDB (http://www.maizegdb.org/documentation/maizemap/ssr_protocols.php).

Material Sampling

For the genotypes tested (sugary maize line rich in anthocyanins and his colourless control) in the 2012 field season about 300 plants were grown in adjacent rows, under the same agronomic conditions, in the experimental field of the University of Milan, Italy (N 45°18′, E 9°15). These plants were selfed (using paper bags) and then harvested at the same time at the end of the season.

About 50 ears were shelled and the seeds obtained mixed to create a single bulk used for the analyses.

Seed treatments

With the aim to mimic the processing treatments used for the sweet corn already available at the supermarket, we decided to test the uncoloured control seeds and the new coloured ones raw and after 2 different cooking treatments (100 seeds each). The steam cooking method involved a mild cooking of 10 minutes during which no contact between the seeds and the boiling water occurred. Other seeds underwent an autoclave cycle, consisting of 20 minutes of a constant pressure of 1 atm and a constant temperature of 120°C. After these treatments the seeds were analysed as described below.

Metabolite quantification (Anthocyanins, flavonols and phenolic acids quantification)

A pool of 10 seeds per treatment – raw, steam cooked and autoclaved - and per line was used to extract flavonoid metabolites. The seeds were ground in a mortar with the extraction buffer (1% HCl, 95% ethanol) in the presence of quartz sand. A sequence of consecutive washing steps of 30 minutes were performed until the extraction buffer turned out to be transparent. Finally the collected supernatants underwent a centrifugation at 13000rpm for 30 minutes, and then were used to spectrophotometrically to determine anthocyanins at λ=530 nm, flavonols at λ=350 nm and phenolic acids at λ=280 nm.

The amount of anthocyanins was calculated as cyanidin-3-glucoside equivalents (molar extinction coefficient (e) 26900 L m^{-1} mol^{-1}, MW 449.2), flavonoids as quercitin-3-glucoside

equivalents (molar extinction coefficient (e) 21877 L m^{-1} mol^{-1}, MW 464.38) and phenolic compounds as ferulic acid equivalents (molar extinction coefficient (e) 14700 L m^{-1} mol^{-1}, MW 194.18) for 100 g of seed weight.

The analyses were conducted on four seeds bulk (10 seeds each) randomly selected for each type. The confidence interval (C.I.) at 95% was calculated.

Qualitative determination of anthocyanins: TLC

The pericarp layer of 2 kernels per treatment of coloured and uncoloured lines were excised and boiled at 100°C with 2 ml of 2N HCl for 40 minutes. After adding 1 ml of isoamyl alcohol, the upper phase was dried and dissolved in EtOH 95% and HCl 1%. The standards of cyanidin, pelargonidin and delphinidin were loaded on a pre-coated plastic sheet (POLYGRAM CEL 300, MACHEREY-NAGEL) for Thin Layer Chromatography (TLC) together with the samples to be tested. The solvent used for the TLC running was formic acid:HCl:water 5:2:3. The developed plates were pictured with a digital camera (A430 Canon) using both white and UV illumination.

Antiradical ability assay

The free radical-scavenging activity was tested using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Brand-Williams et al. 1995; Cevallos-Casals and Cisneros-Zevallos 2003; Leong and Shui 2002; Hu et al. 2004; Yang and Zhai 2010). Five coloured sugary seeds per each treatment were excised from the pericarp; the same procedure was also followed for the uncoloured untreated seeds. The pool of 5 pericarps for each treatment was ground with liquid nitrogen. An adequate aliquot was extracted with acetone 70% (acetone:water 70:30 v/v) according to the ratio 1:8 (w/v) for 3 hours. Then the samples were centrifuged for 10 minutes at 13000rpm and the colored extracts were equalized with a dilution based on the anthocyanin content of each treatment.

Then a 0.12 mM DPPH ethanolic solution was added to increasing aliquots of each sample extract, conveniently diluted. The final volumes of 2.5 ml of these preparations were left 1 hour in the dark at room temperature before the discoloration absorbance was spectrophotometrically recorded at 516 nm.

The percentage of the scavenged DPPH was calculated as:

$$\% \text{ DPPH} = (Ac - As) \times 100 / Ac$$

where Ac is the absorbance of the control, and As is the absorbance of each increasing aliquot of the sample (Leong and Shui 2002; Hu et al. 2004; Yang and Zhai 2010). Finally the amounts of the increasing aliquots of each extract were interpolated with the corresponding DPPH scavenged percentage, tracing the reported curve.

Panel Test

To test the acceptability of the new coloured sugary line, 12 blinded subjects were randomly recruited and asked to taste 4 kernels of both the uncoloured and coloured line. No cooking treatment, nor salt nor dressing were added to the kernels. Each subject expressed his judgment about his appreciation according to a scale from 1, the worst, to 10, the best. The mean, the median and the mode of the judgments relative to the two different kinds of kernels were calculated.

Results

Development of a coloured sugary line

o develop a new coloured sugary line, a tropical black corn plant was used as the source of the genes of the anthocyanins biosynthesis pathway in a backcrossing breeding scheme, while a commercial sugary yellow line was used as the recurrent parent (Fig.1a). Two SSRs markers helped to select the heterozygous plants used for crossing and the homozygous plants during the self-pollination cycles (Fig.1a). In fact the *nc009* SSR marker, part of the *Pl* gene and the *bnlg1064* SSR marker, located next to the *B* gene, were found to be polymorphic between uncoloured and coloured plants (Fig.2). This breeding scheme allowed us to obtain a sugary plant with a pigmented ear, harvested 21 days after pollination (d.a.p.) (Fig.1b). Simultaneously, in the same field in Landriano (PV, Italy), the yellow commercial sugary line was grown and harvested 22 d.a.p. to be used as the control (Fig.1b).

The breeding scheme provided good results: both the fresh and dry mean seed weights did not show significant differences on comparing coloured with non-coloured raw seeds (Fig.1S).

Then for both the coloured and uncoloured sugary lines the seed anthocyanin, flavonol and phenolic acid compounds were spectrophotometrically quantified (Table 1).

The anthocyanin quantification scored a mean amount of 118.92 mg/100g in the raw seeds of the coloured line (Table 1), while no pigment was detected in the corresponding uncoloured commercial line (Table 1). Also for each of the other metabolite classes, the red line scored a significantly higher value compared to the yellow line: 81.04 mg/100g *vs* 31.23 mg/100g of flavonols and 121.67 mg/100g *vs* 52.49 mg/100g of phenolic acids.

Effects of the cooking treatments on anthocyanins, flavonols and phenolic acids content.

The steam treatment caused a small decrease in the anthocyanins content of the new coloured line: the 118.92 mg/100g amount in the fresh seeds fell to 96.82 mg/100 (Table 1). The effect of the autoclave cycle was dramatic, destroying a large part of the anthocyanins, which reached the final amount of 19.6 mg/100g (Table 1). Strikingly neither the flavonols nor the phenolic acids were degraded by the steaming procedure, on the contrary this treatment caused a significant increase in the flavonol amounts (Table 1). This increase seems to be higher for the coloured seeds (81.04 mg/100g before the treatment and 115.28 mg/100g after) than for the uncoloured ones (31.32 mg/100g before the treatment and 39.51 mg/100g after) (Table 1). The same pattern was found for the phenolic acids, with the red line scoring 81.043 mg/100g before and 156.66 mg/100g after the treatment (Table 1) and the yellow line 31.23 mg/100g before and 64.07 mg/100g after the steam treatment (Table 1).
The autoclave cycle led to a marked decrease in flavonols and phenolic acids content in the red line (Table 1), while this decrease was less evident in the yellow line (Table 1).

Qualitative analysis of anthocyanins

To understand whether the cooking treatments modified the chemical structure of the leftover anthocyanins, a TLC was performed comparing the extracts of raw and treated seeds uncoloured and coloured. The plate in Fig.3a showed the spots corresponding to the 3 standards delphinidin, cyanidin and pelargonidin (lanes 1-3), then the 3 coloured samples – raw, steam cooked and autoclaved- (lanes 4-6) and finally the 3 uncoloured samples (lanes 7-9). Cyanidin was the most abundant anthocyanin in the 3 coloured samples, while no spots corresponding to the standards were identified in the uncoloured samples. The extract obtained from the raw seeds (lane 4), also revealed the presence of

pelargonidin, less abundant than cyanidin, and another spot, not identified by the standards. The same pattern even if less intense, is shown by the coloured steam cooked sample (lane 5). The spots relative to the coloured autoclaved sample were very weak (lane 6), so that only the cyanidin is visible. The UV picture of the TLC plate revealed another unidentified spot (Fig.3b), not detected in visible light, and present in both the coloured autoclaved sample (lane 6) and the uncoloured untreated sample (lane 7). This spot was also present, even if weaker, in the uncoloured steam cooked and autoclaved samples too (lanes 8-9).

DPPH Scavenging ability

The diagram representing the DPPH scavenging ability clearly showed that the new coloured sugary line has a much higher antioxidant activity compared to the uncoloured sample (Fig4a). After the equalization of the extracts among the three different treatments, through suitable dilutions based on the anthocyanins content, the curves of the raw and the steam cooked coloured samples are characterized by a similar tendency (Fig.4b), while the autoclaved coloured extract showed a lower radical scavenging ability (Fig.4b).

Panel Test/ consumer test

Twelve blinded subjects, randomly chosen, were asked to express a judgment about the acceptability of the new coloured sugary corn and of the respective control (Table 2). Both lines were tested without cooking, salt and dressing.
The acceptability mean scores were 6.75 for both lines (Table 2), attesting no significant differences between the acceptability for taste alone of the traditionally uncoloured and the new coloured sugary products (Table 2).

Discussion

Sugary corn is a well-established product in the market and a very popular ingredient in the diet especially in the USA. Some reports showed that dietary consumption of sweet corn seems to be able to inhibit tumour growth in mice (Tokuji et al. 2009), probably because of the presence of phenolic compounds, particularly ferulic acid (Tokuji et al. 2009). The ability of some molecules to prevent several chronic diseases such as cancer is supposed to originate from their antioxidant potential (Virgili and Marino 2008). In maize

the antioxidant potential could be increased thanks to its capacity to accumulate anthocyanins in the kernels. In fact anthocyanins are antioxidant molecules whose regular consumption is associated with a high number of health benefits. Therefore improving sweet corn by increasing the anthocyanins content could lead it to being considered as a functional food. For this purpose a recurrent breeding scheme was planned (Fig 1a). A tropical black corn plant bearing the *Pl* and *B* regulatory genes, required to activate the anthocyanin accumulation in the seed pericarp, was used as source of the genes for the pigment biosynthesis, while a commercial sugary yellow line was used as the recurrent parent (Fig.1a). The selection procedure was based on the use of 2 molecular markers, *nc009* and *bnlg1064*, polymorphic for the *Pl* and *B* genes between the parents of the cross (Fig.2). The result of this breeding scheme is a coloured sugary plant, characterized by the genetic background of the commercial uncoloured sugary line with the exception of the presence of the anthocyanin regulatory genes in the dominant form (Fig. 1b). The new coloured sugary line was then analysed using the uncoloured commercial sugary isogenic line as control.

The coloured and the uncoloured sweet corn lines are near-isogenic lines, and as a consequence a near-isogenic food, that differs only in the content of specific phytonutrients and thus appears to be an useful tool to reduce the complexity of the studies about the diet-health relationship (Martin et al. 2011).

Sweet corn is harvested before the time of field maize physiological maturity, at about 20-21 d.a.p. The fresh and dry seed weights did not show significant differences between the two isogenic lines (Fig.1S), attesting to the good result coming from the breeding work. The introgression of the colour genes allowed us to obtain a red sugary line able to accumulate 118.92±14.97 mg/100g of anthocyanins in the fresh kernels (Table 1), while no pigment was detected in control sugary kernels (Table 1). This appeared to be a good amount in comparison with berries that accumulate 25 to 698 mg/100g (Mazza and Miniati 1993; Wang and Lin 2000; Wu et al. 2006; Koponen et al. 2007), black rice, that accumulates 10-493 mg/100g (Ryu et al. 1998) and coloured popcorn, that accumulates around 36-66.44 mg/100g (Lago et al. 2013). In addition to anthocyanins, sweet corn is also able to synthesize phenolics compounds, particularly ferulic acid (Balasubashini et al. 2003; Tokuji et al. 2009). Ferulic acid is synthesized starting from phenylalanine following the phenylpropanoids pathway. Therefore phenolic acids share a part of the biosynthetic way with anthocyanins and with flavonols, the most abundant group of flavonoids

among plants, proven to have many human health beneficial effects (Formica and Regelson 1995; Duthie et al. 2000). So we quantified the amount of phenolic acids and flavonols in both coloured and uncoloured sweet corn lines. The results showed a significantly higher amount of both in the new coloured sugary line, than in the control uncoloured one (Table 1). This could be expected because these classes of molecules share the first part of the biosynthesis pathway with the anthocyanin pathway, so that the active alleles of the anthocyanin regulatory genes could have pushed up the quantities of all the structural genes of the flavonoids biosynthesis. Therefore the presence of the anthocyanin pigments in the new coloured sugary line is a nodal point because they also seem to boost the amounts of other flavonoids and health-promoting compounds too: the anthocyanin presence makes the new sugary coloured line a good candidate as an everyday functional food in the diet of many people. The DPPH scavenging ability test seems to strengthen this hypothesis (Fig.4a): the raw uncoloured commercial sugary seed extract showed a much lower antioxidant ability compared to the raw coloured one, attesting the anthocyanins' remarkable power (Fig.4a). This is in agreement with previously reported data about a coloured popcorn line (Lago et al. 2013), consequently the coloured sweet corn can be consider a new functional food.

However while part of the sweet corn crop is consumed as fresh grains or fresh ears, most of it is consumed as processed canned sweet corn (Dewanto et al. 2002). Some of the thermal procedures required for sweet corn processing are known to lower the nutritional level of grains and vegetables in comparison with the fresh ones (Lathrop et al. 1980; Rao et al. 1981; Burge et al. 1995; Murcia et al. 2000; Dewanto et al. 2002). Therefore it is important to understand the effect of sweet corn processing on the anthocyanin molecules, at both quantitative and qualitative level. Big companies, e. g. Bonduelle and Conserve Italia in Italy and Allens in USA, studied the best methods for thermal processing and conservation of canned food: first of all small amounts of salt water and sugars were added to the kernels inside the can, where the vacuum is imposed. Then the product underwent a steam cooking, but the presence of the vacuum allowed a lowering and shortening of the heating procedure, so that the kernels are subjected only to a sterilization and not to a proper cooking. As a consequence the vegetable can keep its flavour and its nutritional properties. The correct balance between vacuum and temperature is often held as a trade secret by the companies (http://www.bonduelle.it/la-cottura-al-vapore-secondo-bonduelle/ accessed 26 august

2013). For this reason we decided to subject the 2 sweet corn lines to different cooking processes: a mild cooking with steam and a severe one with the autoclave. The steam cooking treatment seems to only slightly decrease the anthocyanins amount (Table 1), as already found by Vallejo et al. (2003). The autoclave cooking on the other hand resulted in a more dramatic effect causing the reduction of the anthocyanins level by about 83% in comparison with the untreated kernels. This result was in agreement with previous data reporting that the stability of anthocyanins in cooked foods is dependent on the temperature and on the heating time of the thermal process (Abdel-Aal et al. 2003; Cabrita et al. 2000; Hiemori et al. 2009). The big difference in the degrading ability of the cooking processes used could be explained by the fact that the steam cooking was not only milder but also shorter than the autoclave treatment so that it was able only to inactivate some oxidative enzymes and not to destroy the pigments that are present in the edible part of the vegetable (Howard et al. 1994; Vallejo et al. 2003).

Moreover steam cooking seems to increase flavonols and phenolic acids in both the coloured (+ 42.25% and + 28.76% respectively) and the uncoloured line (+26.51% and + 22.06% respectively) (Table 1).

The autoclave cooking caused an increase of 9.75% for the flavonols and 7.44% for the phenolic acids in the coloured kernels (Table 1), and of 94.46% and of 71.40% respectively in the uncoloured ones (Table 1).

This was in agreement with the results of Dewanto et al. (2002) who found that the free phenolic portion in their sweet corn significantly increased after the thermal process. This can be explained by the fact that the heating causing the breakdown the cellular constituents, allowed the release of the bound phenolic acids portion (Dewanto et al. 2002).

At this point it was important to understand whether the cooking was able to change the structure of the pigments and consequently the antioxidant ability of the leftover anthocyanins, not degraded by the heating. With this purpose the DPPH assay was also performed on the extracts coming from the raw, the steam cooked and from the autoclaved coloured kernels, equalized through proper dilutions on the basis of the anthocyanin amount. Anthocyanin amounts being equal among them, the raw and steamed kernels showed the same scavenging ability (Fig.4b), attesting that no structural changes occurred in the leftover pigment molecules after the steam cooking. On the contrary the extract obtained from the autoclaved kernels had a much lower antioxidant

power (Fig.4b). This could be caused by the strong treatment of the autoclave, in contrast to the lighter one of the steam treatment: probably one hour of heating coupled with the high pressure was able to degrade not only the anthocyanin molecules but also some other antioxidant compound., such as for example vitamin C (Burge and Fraile 1995; Murcia et al. 2000; Dewanto et al. 2002) or β-carotene and lutein carotenoids (Kurilich and Juvik 1999; Tokuji et al. 2009).

To confirm that no structural or chemical changes in anthocyanin molecules occur after the thermal processes, Thin Layer Chromatography was performed (Fig.3). The spots of the coloured samples clearly showed that the anthocyanin aglycons did not change their structure following cooking, only their amount decreased (Fig.3a). We noticed the presence of a little spot above the pelargonidin one (Fig.3a): it is not present in other *B/Pl* maize genotypes, such as the coloured popcorn (Lago et al. 2013). This could be explained by the fact that sweet corn is a fresh product, composed by developing kernels that are still accumulating pigments in the pericarp; therefore the metabolite profile is not definitive as in the dry maize kernels. Deeper and more precise analyses are needed to finely characterize the metabolite profile of this coloured line. However this study suggested that the new coloured sugary line is a good source of anthocyanins, of other beneficial flavonoids and of antioxidant potential, and thus it can be considered a good functional food. Our results also suggest that to preserve the healthy properties of the coloured sweet corn it is better to consume it fresh but if processing is needed it would be better to use a mild process, such as the steam treatment, in order to benefit from the best nutritional composition.

In the meantime the acceptability of the new product in comparison to the uncoloured one was tested on 12 blinded subjects, randomly chosen (Table 2). The kernels were tested with no cooking, no salt and no dressing in order to level the taste. The appreciation scores did not show significant differences between the uncoloured and coloured sugary kernels (Table 2), suggesting that the healthier properties due to the pigment presence could persuade the consumer to prefer the coloured sweet corn to the uncoloured one.

Acknowledgments

We wish to thank Dr. Davide Reginelli for his hard work in the field and Dr. Lesley Currah for her precious suggestions.

Figure and Tables

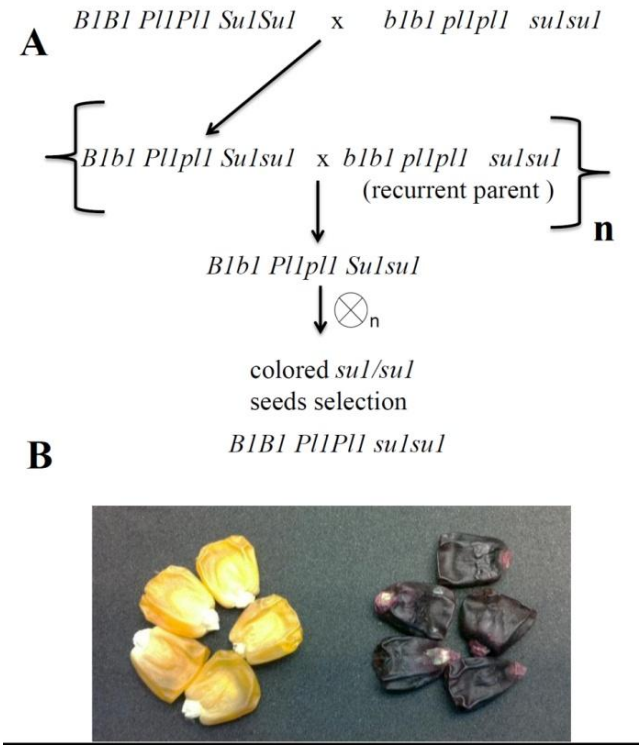


Figure 1 A: Recurrent Selection Scheme: the cross between the *B1Pl1* line, source of the regulatory biosynthetic genes and the commercial uncoloured sugary corn gave rise to heterozygous plants for the *B- Pl-* genes. Among them, the highest anthocyanin content plants were selected for the backcrossing with the recurrent parent. Then the best plants underwent some cycles of self-pollination. B: Phenotype of uncoloured (left) and coloured (right) sweet corn kernels

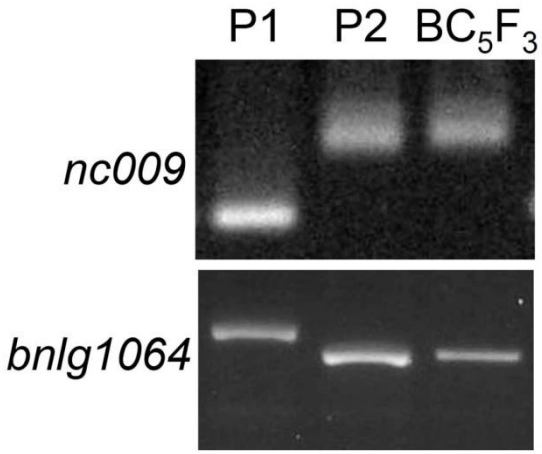


Figure 2. Molecular Assisted Selection: the *nc009* SSR, part of the *Pl1* gene and the *bnlg1064* SSR, next to the *B1* gene, was found to be polymorphic between the coloured and the colourless parents. The heterozygous individuals were easily detected and selected to carry on the breeding selection scheme. P1, sugary corn line; P2 *B1Pl1* line; BC5F3, coloured sugary corn line developed

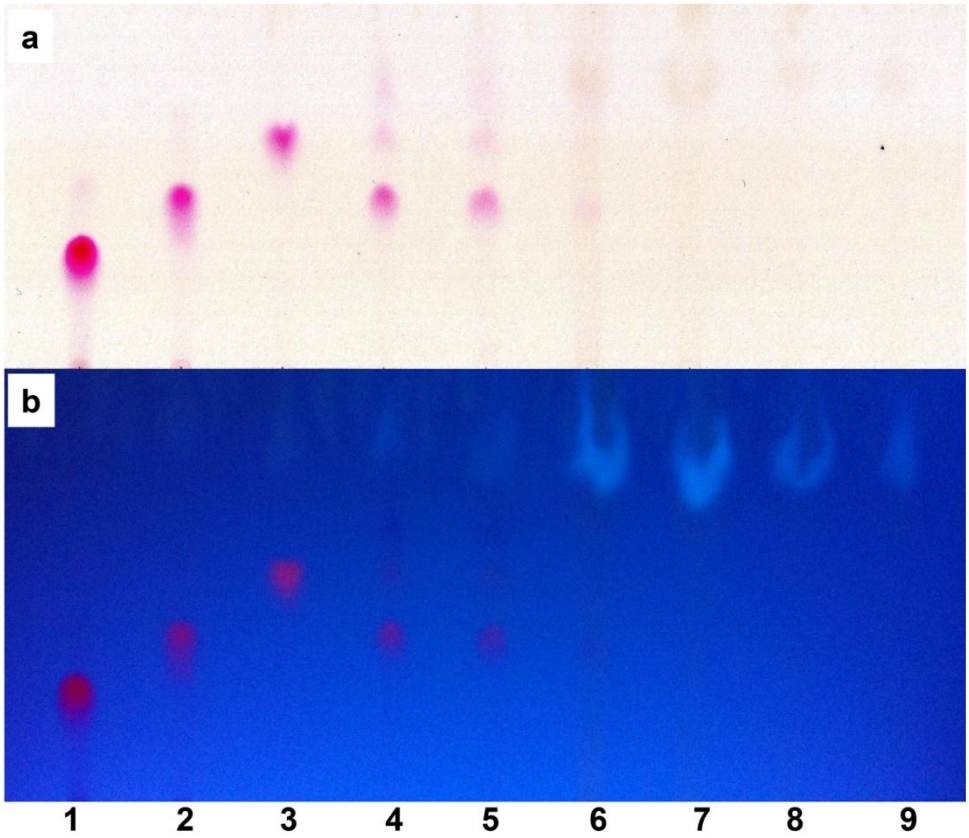


Figure 3. Pictures of the TLC plate taken under visible (a) or UV (b) light. The spots represent: from lane 1 to 3, the delphinidin, cyanidin and pelargonidin standards from lane 4 to 6 the anthocyanin extracts coming from the coloured raw steamed cooked and autoclaved kernels, while the respective uncoloured controls are represented in lanes 7 to 9.

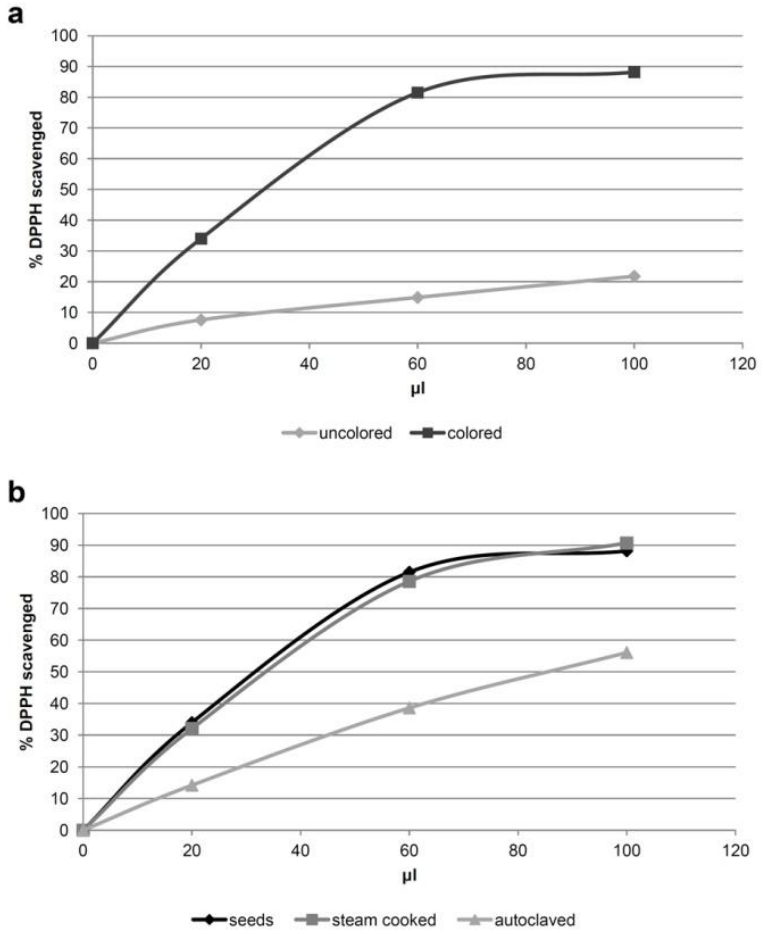


Figure 4. Comparison of the antioxidant ability in the DPPH radical scavenging assay of the coloured raw vs the uncoloured raw seed extracts (a) and of the raw vs steam cooked vs autoclaved coloured seed extracts (b), equalized and diluted according to the anthocyanins concentration

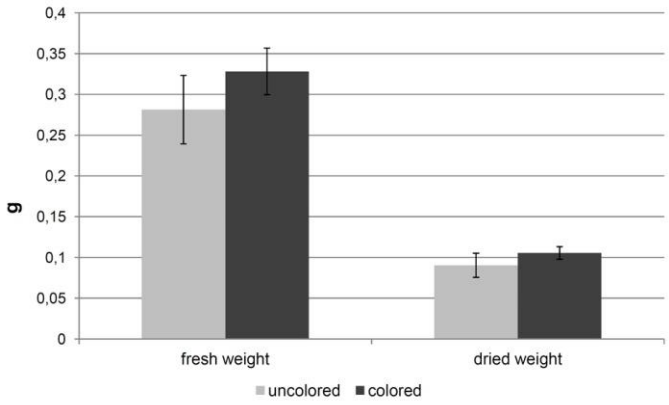


Figure 1S. Comparison of the mean seed weight of the fresh and dried kernels of the uncoloured control line with the new coloured sugary line

Table 1: Spectrophotometric quantification of anthocyanins, flavonols and phenolic acids content in the raw, steam cooked and autoclaved kernels of uncoloured and coloured lines, The confidence intervals at 95% are shown.

		raw	steam cooked	autoclaved
anthocyanins	*uncoloured*	0.23±0.24	0.57±0.70	0.22±0.15
	coloured	118.92±14.97	96.82±2.21	19.6±1.75
flavonols	*uncoloured*	31.23±5.24	39.51±4.8	60.73±9.25
	coloured	81.04±14.54	115.28±2.61	88.94±9.11
phenolic acids	*uncoloured*	52.49±5.68	64.07±2.68	89.97±2.63
	coloured	121.67±25.67	156.66±3.34	130.72±3.97

Table 2: Mean, mode and median about the scores of the panel test, relative to the acceptability degree of the new coloured sugary kernels compared to the commercial uncoloured one, in a randomly blinded group of subjects.

Acceptability Degree		
	uncoloured	**coloured**
mean	6.75	6.75
mode	7	7
median	7	7

References

Abdel-Aal el-SM, Hucl P (2003) Composition and stability of anthocyanins in blue-grained wheat. J Agric Food Chem 51:2174–2180

Aherne SA, O'Brien NM (2002) Dietary flavonols: chemistry, food content, and metabolism. Nutrition 18:75– 81

Balasubashini MS, Rukkumani R, Menon VP (2003) Protective effects of ferulic acid on hyperlipidemic diabetic rats. Acta Diabet 40(3):118–22

Bodeau JP, Walbot V (1992) Regulated transcription of the maize *Bronze-2* promoter in electroporated protoplasts requires the *C1* and *R* gene products. Mol Gen Genet 233:379-387

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. Lebensm Wiss Technol 28:25-30

Brester GW,. http://www.agmrc.org/commodities__products/grains__oilseeds/corn_grain/. Accessed on 24 June 2013

Bülent Coşkun M, Yalçin I, Özarslan C (2006) Physical properties of sweet corn seed (*Zea mays saccharata* Sturt.). J Food Eng 74:523–528

Burge P, Fraile P (1995) Vitamin C destruction during the cooking of a potato dish. Lebensm.-Wiss. Technol. 28:506-514

Cabrita L, Fossen T, Andersen OM (2000) Color and stability of the six common anthocyanidin 3-glucosides in aqueous solution. Food Chem 68:101–107

Castilla P, Dávalos A, Teruel JL et al 2008) Comparative effects of dietary supplementation with red grape juice and vitamin E on production of superoxide by circulating neutrophil NADPH oxidase in hemodialysis patients. Am J Clin Nutr 87:1053–1061

Cevallos-Casals BA, Cisneros-Zevallos L (2003) Stoichiometric and kinetic studies of phenolic antioxidants from Andean purple corn and red-fleshed sweetpotato. J Agric Food Chem 51:3313-3319

Chandler VL, Radicella JP, Robbins Chen JC, Turks D (1989) Two regulatory genes of the maize anthocyanin pathway are homologous: isolation of *B* utilizing *R* genomic sequences. Plant Cell 1:1175–1183

DeFuria J, Bennett G, Strissel KJ, Perfield JW 2nd. et al (2009) Dietary blueberry attenuates whole-body insulin resistance in high fat-fed mice by reducing adipocyte death and its inflammatory sequelae. J Nutr 139:1510–1516

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19-21

Dewanto V, Wu X, Liu RH (2002) Processed sweet corn has higher antioxidant activity. J Agric Food Chem 50:4959–4964

Dooner HK, Robbins TP, Jorgensen RA (1991) Genetic and developmental control of anthocyanin biosynthesis. Annu Rev Genet 25:173–199

Duthie G, Duthie SJ, Kyle JAM (2000) Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. Nutr Res Rev 13:79-106

Formica JV, Regelson W (1995) Review of the biology of quercetin and related bioflavonoids. Food Chem Toxicol 33:1061-1080

Gaut BS (2001) Patterns of chromosomal duplication in maize and their implications for comparative maps of the grasses. Genom Res 11:55-66

Giusti MM, Wrolstad RE (2003) Acylated anthocyanins from edible sources and their applications in food systems. Biochem Eng J 14:217-225

Goyarzu P, Malin DH, Lau FC, Taglialatela G et al (2004) Blueberry supplemented diet: effects on object recognition memory and nuclear factor-kappa B levels in aged rats. Nutr Neurosci 7:75–83

Haase H, Overbeck S, Rink L (2008) Zinc supplementation for the treatment or prevention of disease: current status and future perspectives. Exp Gerontol 43:394–408

Hansen R, content specialist, AgMRC, Iowa State University, Sweet corn profile http://www.agmrc.org/commodities__products/grains__oilseeds/corn_grain/

Haynes C, Eldon E, Richard J, Sweet Corn, Iowa State University Horticulture Guide

http://www.maizeg db.org/ssr.php
http://www.maizegdb.org/documentation/maizemap/ssr_protocols.php

Hiemori M, Koh E, Mitchell AE (2009) Influence of cooking on anthocyanins in black rice (*Oryza sativa* L. *japonica* var. SBR). J Agric Food Chem 57(5):1908-14

Howard LR, Griffin LE, Lee Y (1994) Steam treatment of minimally processed tuber sticks to control surface discoloration. J Food Sci 59:356–358

Hu FL, Lu RL, Huang B, Ming L (2004) Free radical scavenging activity of extracts prepared from fresh leaves of selected Chinese medicinal plants. Fitoterapia 75:14−23

Iwasaki-Kurashige K, Loyaga-Rendon RY, Matsumoto H et al (2006) Possible mediators involved in decreasing peripheral vascular resistance with blackcurrant concentrate (BC) in hind-limb perfusion model of the rat. Vasc Pharmacol 44: 215–223

Koponen JM, Happonen AM, Mattila PH, Torronen AR (2007) Contents of Anthocyanins and Ellagitannins in Selected Foods Consumed in Finland. J. Agric Food Chem 55:1612-1619

Kurilich AC, Juvik JA (1999) Quantification of carotenoid and tocopherol antioxidants in *Zea mays*. J Agric Food Chem 47:1948–55

Lago C, Landoni M, Cassani E, Doria E, Nielsen E, Pilu, R (2013) Study and characterization of a novel functional food: purple popcorn. Mol Breeding 31:575–585

Lathrop PJ, Leung HK (1980) Rates of ascorbic acid degradation during thermal processing of canned peas. J Food Sci 45:152-153

Lau FC, Bielinski DF, Joseph JA (2007) Inhibitory effects of blueberry extract on the production of inflammatory mediators in lipopolysaccharide-activated BV2 microglia. J Neurosci Res 85:1010–1017

Leong LP, Shui G (2002) An investigation of antioxidant capacity of fruits in Singapore markets. Food Chem 76:69-75

Martin C, Butelli E, Petroni K, Tonelli C (2011) How can research on plants contribute to promoting human health? The Plant Cell 23:1685–1699

Matsumoto H, Kamm KE, Stull JT, Azuma H (2005) Delphinidin-3-rutinoside relaxes the bovine ciliary smooth muscle trough activation of ETB receptor and NO/cGMP pathway. Exp. Eye Res 80:313–322

Mazza G, Miniati E (1993) Anthocyanins in fruits, vegetables, and grains. CRC Press, Boca Raton, FL

Murcia MA, Lopez-Ayerra B, Martinez-Tome M, Vera AM, Garcia-Carmona F (2000) Evolution of ascorbic acid and peroxidase during industrial processing of broccoli. J Sci Food Agric 80:1882-1886

Peng CH, Liu LK, Chuang CM et al (2011) Mulberry water extracts possess an anti-obesity effect and ability to inhibit hepatic lipogenesis and promote lipolysis. J Agr Food Chem 59:2663–2671

Pilu R, Piazza P, Petroni K, Ronchi A, Martin C, Tonelli C (2003) *pl-bol3*, a complex allele of the anthocyanin regulatory *pl1* locus that arose in a naturally occurring maize population. Plant J 36:510–521

Price KR, Rhodes MJC (1997) Analysis of the major flavonol glycosides present in four varieties of onion and changes in composition resulting from autolysis. J Sci Food Agric 74:331-339

Prior RL, Wu X, Gu L et al. (2008) Whole berries versus berry anthocyanins: interactions with dietary fat levels in the C57BL/6J mouse model of obesity. J Agr Food Chem 56:647–653

Rao MA, Lee CY, Katz J, Cooley HJ (1981) A kinetic study of the loss of vitamin C, color, and firmness during thermal processing of canned peas. J Food Sci 46:636-637

Rissanen TH, Voutilainen S, Virtanen JK et al (2003) Low intake of fruits, berries and vegetables is associated with excess mortality in men: the Kuopio Ischemic Heart Disease Risk Factor (KIHD) Study. J Nutr 133:199–204

Ryu SN, Park SZ, Ho CT (1998) High performance liquid chromatographic determination of anthocyanin pigments in some varieties of black rice. J Food Drug Anal 6:729–736

Seymour EM, SK Lewis, Urcuyo-Llanes DE et al (2009) Regular tart cherry intake alters abdominal adiposity, adipose gene transcription, and inflammation in obesity-prone rats fed a high fat diet. J Med Food 12:935–942

Shukitt-Hale B, Lau FC, Carey AN et al (2008) Blueberry polyphenols attenuate kainic acid-induced decrements in cognition and alter inflammatory gene expression in rat hippocampus. Nutr Neurosci 11:172–182

Stewart A, Bozonnet S, Mullen W et al (2000) Occurrence of flavonols in tomato and tomato-based products. J Agric Food Chem 48:2663-9

Titta L, Trinei M, Stendardo M et al (2010) Blood orange juice inhibits fat accumulation in mice. Int J Obesity 34:578–588

Topping DL, Clifton PM (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev 81:1031–64

Tokuji Y, Akiyama K, Yunoki K, inoshita M, Sasaki K, Kobayashi H, Masahirowada, Ohnishi A (2009) Screening for Beneficial Effects of Oral Intake of Sweet Corn by DNAMicroarray Analysis. J Food Sci 74(7): H197-203

Tsuda T (2008) Regulation of adipocyte function by anthocyanins; possibility of preventing the metabolic syndrome. J Agric Food Chem 56:642–646

Tsuda T (2012) Dietary anthocyanin-rich plants: biochemical basis and recent progress in health benefits studies. Mol Nutr Food Res 56:159-170

USDA, Foreign Agricultural Service, World Corn Production, Consumption, and Stocks

Vallejo et al (2003) Phenolic compounds in cooked broccoli. J Sci Food Agric 83:1511–1516

Virgili F, Marino M (2008) Regulation of cellular signals from nutritional molecules: A specific role for phytochemicals, beyond antioxidant activity. Free Radic Biol Med 45:1205–1216

Wang SY, Lin HS (2000) Antioxidant Activity in Fruits and Leaves of Blackberry, Raspberry, and Strawberry Varies with Cultivar and Developmental Stage. J Agric Food Chem 48:140-146

Yang Z, Zhai W (2010) Identification and antioxidant activity of anthocyanins extracted from the seed and cob of purple corn (*Zea mays* L.). Innov Food Sci Emerg 11:169–176

Žilić S, Serpen A, Akıllıoğlu G, Gökmen V, Vančetovic J (2012) Phenolic Compounds, Carotenoids, Anthocyanins, and Antioxidant Capacity of Colored Maize (*Zea mays* L.) Kernels. J Agric Food Chem 60:1224−1231