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### Micronutrient and functional compounds biofortification of maize grains

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# ACCEPTED MANUSCRIPT

## Micronutrient and functional compounds biofortification of maize grains

### Short title: Biofortification of maize grains

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## Micronutrient and functional compounds biofortification of maize grains

### Abstract

Maize, in addition to being the main staple food in many countries, is used in the production of hundreds of products. It is rich in compounds with potential benefits to health, such as carotenoids, phenolic compounds, vitamin E and minerals that act as cofactors for antioxidant enzymes. Many of these compounds have been neglected thus far in the scientific literature. Nevertheless, deficiencies in the precursors of vitamin A and some minerals, such as

iron and zinc, in maize, in association with the great genetic variability in its cultivars and our genomic, transcriptomic and metabolomic knowledge of this species make targeted biofortification strategies for maize promising. This review discusses the potential of the main micro-constituents found in maize with a focus on studies aimed at biofortification.

**Key words:** grain, antioxidants, minerals, endogenous fortification

## Introduction

The three most commonly grown grains (maize, wheat and rice) provide more than half of the caloric needs of humans. Maize and wheat grains are also commonly eaten by animals. Maize alone is responsible for providing 15% of the protein and 20% of the calories in the human diet, and this crop covered a cultivated area of 159.5 million hectares in 2009 (Faostate, 2009). The importance of this crop is demonstrated by the multiple ways it is exploited. For example, maize is consumed in a variety of forms, serving as the raw material for the production of at least one hundred products, and maize derivatives are used in industrial applications ranging from textiles to oil to pharmaceuticals.

Cereals such as maize have a matrix rich in organic compounds and minerals with potential benefits to health. These compounds may act as antioxidants (carotenoids and phenolic compounds), as cofactors for antioxidant enzymes (selenium, zinc, manganese and copper) or as indirect antioxidants (betaine, choline and folate) (Hänsch and Mendel, 2009). Epidemiological studies have shown that the consumption of whole grains and grain products is preventative against cardiovascular disease (Liu et al., 1999; Anderson et al., 2000), some types of cancer (Kim et al., 2001), type 2 diabetes (Liu et al., 2000) and obesity (Misra et al., 2009).

However, little attention has been paid to the consumption of grain compared to vegetables, although grains and their products are used as the base of nutritional pyramids to emphasize their importance in the diet. Most publications indicate fruits and vegetables as the main sources of phytochemical compounds (Sommerburg et al., 1998; Haleem et al., 2008). However, grains contain phytochemicals that complement those found in fruits and vegetables (Liu, 2007). While fruits and vegetables contain the vast majority of phytochemical compounds in glycoside conjugated forms (free or soluble), the phytochemicals in grains can exist in both free and soluble conjugated forms as well as in insoluble complexes. These insoluble complexes are bound to materials, especially from the cell wall; up to 70% of the total phenolic compounds present in maize are found in this form (Bunzel et al., 2001; Serpen et al., 2007). This cell wall material is difficult to digest in the gastrointestinal tract and therefore reaches the colon, where it can be absorbed and act locally, as reported by Adom and Liu (2002) in a study on the effects of cereal grains in colon cancer.

However, there are reasons to believe that the concentrations of many secondary metabolites of plants (phytochemical compounds), in the human diet, are lower than the optimum needed to promote improvements in human health (Brandt and Molgaard, 2001). Thus, while productivity remains the major focus for most breeding programs and producers, grain quality is the most important factor for most cereals due to their levels in the diet. The importance of maize in the diet, especially for populations that use maize as their main staple food; the benefits to health due to the ingestion of functional compounds such as those found in maize; and the need to reduce the deficiencies of some minerals and pro-vitamin A in maize, make this a crop of great interest for biofortification strategies (Ortiz-Monasterio et al., 2007;

Zhu et al., 2007). Biofortification is a term that has been used to cover the four most frequently used endogenous fortification strategies: conventional breeding, mutagenesis, genetic modification and the use of fertilizers (Cakmak, 2008).

As a result, maize is one of the plant species that has been well studied in terms of its genomic and hereditary traits; and the genome sequence of maize are now available (Schnable et al., 2009). Among the Poaceae, only rice exhibits more complex structural and functional genomics, mainly due to the smaller size of the genome (Sasaki and Burr, 2000). The germplasm of maize is composed of DNA from adapted populations, introduced exotic materials and landraces (local strains), and is characterized by great genetic variability (Araújo and Nass, 2002) with an excellent potential for improvement. As consumer markets become more demanding with regard to nutritional and functional foods, genetic variability becomes a key factor with the potential to increase compounds in plantas that have the potential to provide human nutrition.

The creation of new varieties is a powerful tool to enhance nutritional compounds of interest (Ortiz-Monasterio et al., 2007) and to increase plant resistance to pathogens and pests (Fujimori et al., 2004). Conventional breeding based on the natural genetic variability of maize has been the most widely used process to develop varieties, but it is also the slowest. Thus, mutagenesis and transgenic techniques have become increasingly common, as they allow targeted action and enable a greater understanding of biosynthetic routes (Huang et al., 2004; Naqvi et al., 2009). Biofortification through fertilization with nutrients has also been used extensively in maize, and supplemental foliar fertilization is one of the fastest growing of these techniques, mainly because of the development of hybrids with high nutritional potential and the

increasing use of fertilizers containing high concentrations of nutrients and low micronutrient levels. This practice is more common in major commodity crops and may be less expensive, since it is commonly used concurrently with the application of herbicides and/or insecticides. In addition, spraying allows the application of minerals at the appropriate time during plant development, turning this supplemental fertilization efficient (Brakemeier, 1999).

Thus, based on the genetic variability, metabolic vulnerability in the face of abiotic stress, and the important nutritional support provided by maize, this species is a major source of nutrients and functional compounds among plants, which can be improved by biofortification techniques. In this context, this review discusses the potential of the main microconstituents found in maize with a focus on biofortification strategies for these compounds.

## Minerals

Mineral micronutrients, such as zinc (Zn), iron (Fe), copper (Cu) and manganese (Mn), as well as trace elements, including selenium (Se), have important metabolic functions, acting as cofactors for a number of antioxidant enzymes. For example, superoxide dismutase is Zn, Cu and Mn dependent; glutathione peroxidase and thioredoxin reductase are Se dependent; and catalase is Fe dependent (Hänsch and Mendel, 2009). Plants absorb these minerals primarily from the soil and accumulate them in different tissues, including grain. The concentrations of minerals and trace elements vary greatly from one type of cereal to another as well as among the soils where these plants are grown (Curtim et al., 2006; Zhao et al., 2004). However, due to the use of maize as human food and animal feed, increasing productivity, and the purity of industrial fertilizers, the soil has become depleted resulting into loss of mineral phytoavailability (Raut et al., 2010; Lal, 2009). Thus, mineral deficiencies have become a limiting factor in the productivity and

quality of this crop. In addition, some minerals are lost during processing, and others are unavailable in vegetables because they are complexed (specially with poliphenols) or because of the presence of other molecules that interfere with absorption. Thus, despite the importance of these minerals in human health, the concentrations of minerals in plants are comparatively lower than in foods of animal origin, resulting in three billion people exhibiting mineral micronutrient deficiencies (Welch and Graham, 2004), leading to the need to achieve increases in mineral content, especially in cereals such as maize.

In this context, biofortification is a recent strategy that has shown better results than exogenous fortification via supplementation of minerals. However, this strategy requires detailed knowledge of metabolic pathways, uptake, transport and accumulation (Waters and Sankaran, 2010).

One method used for biofortification of minerals is reduction of phytic acid (inositol hexaphosphate) in grain because phytic acid has characteristics of an anti-nutrient due to its ability to chelate trace elements and minerals such as Zn and Fe (Glahn and Wortley, 2002). Chelation causes the minerals to be unavailable for absorption, requiring hydrolysis with the enzyme phytase, which is absent in non-ruminants (Urbano et al., 2000). However, Doria et al. (2009) showed that the maize strain Ipa-1-241, a maize mutant defective in the synthesis of phytic acid during seed maturation, exhibits a low germination capacity compared to the wild-type strain. This grain mutant contains approximately 50% more free or weakly bound iron and presents a high content of free radicals, mainly concentrated in the embryo. In addition, the seed proteins in the mutant were found to be carbonylated, and most exhibited more DNA damage compared to the wild-type strain, whereas the lipids appeared to be more peroxidated, suggesting

a role for phytic acid in protecting seeds against oxidative stress. In this context, the use of phytases during pre-fermentation, such occur in the preparation of bread at pH 5-5.5 (Leenhardt et al., 2005), seems to be a reasonable alternative to reduce phytic acid content and to increase the bioavailability of minerals, especially Fe and Zn (Lopez et al., 2003).

Though phytates were long considered a problem, their nutritional and antioxidant potential is now well established (Rimbach and Pallauf et al., 1998). Phytic acid is located mainly in bran, specifically in the aleurone layer, and is a major phosphorus stock compound in many grains, contributing approximately 70% of the phosphorus (P) content and between 1 and 7% of the dry weight (DW) of grains (De Boland et al., 1975). Furthermore, phytic acid suppresses the oxidative reactions catalyzed by Fe due to its chelating capacity, and it may act as a potent antioxidant *in vivo*, for example, suppressing lipid peroxidation (Graf and Eaton, 1990). Phytic acid can also reduce the incidence of colon cancer and kidney stones as well as protect against inflammatory bowel disease (Graf and Eaton, 1990), in addition to controlling blood glucose levels. Other mechanisms of the action of phytic acid include activation of immunocompetent cells and participation in signal transduction as well as playing roles in cell division and differentiation (Belal et al., 2009).

Thus, taking into account the beneficial effects of phytic acid in plant physiology and human health but also the need to improve mineral intake, an alternative to increasing mineral bioavailability is the ingestion of whole grains, which provide more minerals than processed grains (exhibiting losses of 70% Fe and 75 to 80% Zn and Mn), to offset the deleterious effects of phytic acid. However, there is a need for further studies to investigate the bioavailability of

these minerals and trace elements in whole grains to meet mineral needs and to contribute to the body's antioxidant balance (Liu, 2007).

Other biofortification strategies have focused on directly increasing the mineral content of grains. For example, Zn is essential as a cofactor for transcription and in antioxidant defense and DNA repair, and dietary deficiency of this mineral may contribute to oxidative damage and DNA modifications, which increase the risk of cancer (Mafra, 2005). Maize contains an average of  $20 \text{ } \mu\text{g g}^{-1}$  Zn, corresponding to approximately 40% of the requirement for non-pregnant women and infants after breast feeding (Cakmak, 2008). In addition, approximately half of the world's cultivable land is deficient in Zn for the purpose of food production; these soils are associated with Zn deficiency in humans (Cakmak, 2008). Therefore, several attempts have been made to biofortify maize with Zn.

Conventional breeding is the main strategy adopted by the HarvestPlus program (<http://www.harvestplus.org/>), which aims to biofortify key crops in developing countries through identification of features related to high concentrations of minerals (Zn and Fe) in different seeds (wheat, rice, maize, cassava, millet, beans and sweet potatoes) and transfer of these features to adapted local cultivars (Asia and Africa). This program aims at achieving mineral content levels between 33% and 100%, depending on the mineral and specie; a bean cultivar with a high Fe concentration has already been developed. Similarly, varieties of rice containing 4-5 times more Fe in grain tissues after processing were generated by the International Rice Research Institute (IRRI) (Gregorio, 2002).

In maize germplasm, there are great variations in Zn content, which has been widely explored in studies addressing conventional breeding (Cakmak, 2008; Simic et al., 2009). At the

molecular level, little information is available about the mechanism of Zn (and other micronutrient) accumulation. However, it is assumed that there is a positive correlation between the level of protein and Zn, possibly due to cosegregation of associated genetic factors (Uauy et al., 2006). Therefore, efforts to increase protein could increase levels of Zn.

The use of fertilizers and/or inductors is also an important strategy for mineral biofortification, as it has been shown to improve the absorption and/or translocation of minerals via the xylem, enhancing vegetative growth and yield (Brakemeier, 1999; Hawrylak-Nowak, 2008). Application of urea containing Zn increase Zn and proteins levels in grains. Although further studies are needed to confirm this, Martin-Ortiz et al. (2009) showed that application of NPK (nitrogen, phosphorus and potassium) with Zn lignosulfate may provide maize with an adequate supply of Zn. Moreover, Zn reduces the absorption of P and the accumulation of phytate in grain, which would make Zn more bioavailable in the digestive tract (Hortz and Mcclafferty, 2007). Additionally, plants from seeds with a high Zn content exhibit an increased ability to cope with adverse environmental conditions (Cakmak, 2008). In general, fertilization with Zn has shown better results in poor Zn soil, whereas foliar application of Zn to wheat in a late growth stage increased its concentration in grain by three times. These positive results may occur because Zn is relatively readily absorbed (Cakmak, 2008).

Iron deficiency affects approximately two billion people worldwide (WHO, 2000), which makes it important to consider this mineral in maize biofortification. Iron has low mobility in soil, but foliar fertilizer application (with FeSO<sub>4</sub> and the chelates Fe-EDDHA and Fe-EDTA) improves its absorption (Frossard et al., 2000; Zhu et al., 2007). In addition, the synergism/antagonism observed between minerals can be an effective strategy to increase

mineral content. Application of N, P and/or K, for example, has been shown to increase the absorption of Fe as well as Zn and calcium (Ca) in the soil, except when used excessively, in which case the opposite effect is observed (Frossard et al., 2000). In rice, N application increased concentrations of Fe but had adverse effects on Zn concentrations (Zhang et al., 2008). In turn, fertilization of maize with N increased the levels of P, K, Ca, magnesium (Mg), sulfur (S), Cu, Fe, Mn and Zn (Ferreira et al., 2001).

Quantitative trait loci (QTLs), associated to the accumulation of minerals in grains have been mapped in several cultivars (Stangoulis et al., 2007), which provides molecular markers that can facilitate the improvement of mineral content. One recent study presented a useful source of SSRs and SNP markers in genes that encode transporters for Zn and Fe in maize, which can be used for genetic mapping, association-assisted selection and the development of transgenic maize fortified with micronutrients (Sharma and Chauha, 2008). For example, overexpression of Zn and Fe transporter proteins was associated with an increase in both micronutrients simultaneously. In barley, expression of genes encoding Zn transport proteins (ZIP family proteins) from *Arabidopsis thaliana* increased Zn concentrations (Ramesh et al., 2004). However, absorption of toxic elements, such as cadmium (Cd), can occur concurrently with absorption of essential nutrients, such as Fe and Zn, because both use the same carriers. Thus, efforts to increase the absorption of these nutrients should seek to avoid possible undesirable consequences, like an increase in the accumulation of heavy metals (Palmgren et al., 2008).

Studies of the transcription factor NAC (NAM-B1) from *Triticum dicoccoides* also showed an increase in the concentration of Zn and Fe in grain tissues, possibly due to stimulation

of leaf senescence and remobilization of these minerals to the grain (Uauy et al., 2006). Furthermore, overexpression of the yellow stripe 1 (*ys1*) gene, which encodes nicotianamine aminotransferase, was associated with an increase in the accumulation of Fe (Curie et al., 2001). *Ys1* can also affect the absorption of Zn and Cu (Didonato et al., 2004). Overexpression of the phytase gene from *Aspergillus* (*Phya*) using the endosperm-specific promoter of rice glutelin-1 (*Gt-1*) in combination with increased levels of Fe-binding ferritin protein from soy, increases the levels and bioavailability of Fe in maize (Drakakaki et al., 2005). The increased ferritin levels in the grain result in protection against metal chelators during the digestion process in humans, thus increasing the absorption of Fe (Lonnerdal et al., 2006). Additionally, the recent observation of the influence of β-carotene content on increasing the bioaccessibility of these minerals (Gautama et al., 2010) should generate new strategies to increase the bioavailability of trace minerals.

Calcium, like Zn, is an important cofactor and molecular signal and is essential in the blood coagulation cascade. Calcium deficiency has a profound impact on bone health and may result in rickets or osteoporosis, depending on age (Relea, 1995). Transgenic strategies to increase Ca in plants have focused on increasing the expression of secondary transporters of  $\text{Ca}^{2+}/\text{H}^+$  located in the membrane of the vacuole. The *Arabidopsis* CAX1 secondary transporter, which is important for Ca homeostasis in plant cells, increased the bioavailability of Ca in transgenic potato tubers (Park et al., 2005). More recently, the same authors observed a 25-32% increase in the concentration of Ca in transgenic lettuce compared to non-transgenic controls, without affecting quality parameters, sensory crispness and flavor (Park et al., 2009).

Similarly, selenium, for which cereals are an excellent source, is an essential trace element for the regulation of antioxidant metabolism in plants and animals. It functions as a

cofactor for glutathione peroxidase, an enzyme that protects tissues against oxidative damage and has a suppressive action on cell proliferation (Slavin, 2003). In a study using rats fed two varieties of wheat, one of which was low in Se ( $23 \text{ mg kg}^{-1}$ ), while the other was rich in Se ( $800 \text{ mg kg}^{-1}$ ), glutathione peroxidase activity in plasma, liver and red blood cells was found to be directly correlated with Se content (Ciapellano et al., 1989). The bioavailability of Se from various fractions of cereals was also positive in rats (60% for wheat bran and 100% for wheat flour) and provided increased glutathione peroxidase activity in the liver and red cells at a diet of approximately  $20 \text{ mg kg}^{-1}$  Se (Reeves et al., 2007).

Selection of varieties of maize with a higher content of Se as well as increased use of Se in fertilizers could provide further improvements in the body's antioxidant potential. Fertilization with selenium may be an intervention associated with great results, as sodium selenate is highly mobile, easily absorbed and accumulates in a bioavailable form, selenomethionine, even in grain (Broadley et al., 2006; Fang et al., 2008; Li et al., 2010). Addition of Se to NPK fertilizers has been widely employed in Finland and New Zealand (countries with soils that are naturally low in Se), which has led to the accumulation of Se in grains (Combs, 2001; Gomez-Galera et al., 2010).

There have been no reports of transgenic maize being modified to increase the accumulation of Se thus far. However, due to the chemical similarity of Se to S, several genes involved in the S metabolic pathway have been identified as possible candidates. In the same metabolic pathway, Se is taken up, accumulated and volatilized. Thus, a strategy to inhibit this stage of evaporation would promote Se accumulation without affecting the metabolism of S (Sors et al., 2005).

In this context, although several studies have been conducted using direct and indirect strategies for mineral biofortification, there are still many gaps that remain to be understood, especially in relation to the chemical and molecular mechanisms of absorption and translocation of minerals and possible synergism/antagonism between them. This information will enable more success in efforts to biofortify minerals with important nutritional and functional actions in human health.

## Carotenoids

Carotenoids are the largest group of pigments synthesized by secondary metabolism, with more than 600 structures characterized. They are synthesized by photosynthetic organisms, including plants and, some bacteria and fungi. Carotenoids in feed are responsible for the color characteristics of many birds, insects and marine invertebrates. In plants, they are essential for growth and development and in the process of photosynthesis as well as being protective against photo-oxidative damage and as precursors of abscisic acid (Gallagher et al., 2004). The presence of carotenoids in grains adds nutritional value to the diet, as they are precursors of vitamin A and essential retinoid compounds (Fraser and Bramley, 2004).

Cereals are an important source of carotenoids, with the highest levels present in maize (approximately 11 mg of carotenoids per kilogram of DW), and are uniformly distributed in the grain (Panfili et al., 2004). The carotenoids include  $\alpha$  and  $\beta$ -carotene, cryptoxanthin, zeinoxantina, lutein and zeaxanthin (Figure 1); the last two are mostly xanthophylls found in maize ( $6\text{-}18 \text{ mg g}^{-1}$  of zeaxanthin and  $4\text{-}8 \text{ mg g}^{-1}$  of lutein) (De Oliveira and Rodriguez-Amaya, 2007). These xanthophylls are of interest due to their association with eye health. They are the only carotenoids found in the macula, functioning to protect the eye against free radicals and

blue wavelengths of light from the near ultraviolet (Mrcophth et al., 2008). Studies have also shown that dietary lutein and zeaxanthin can reduce the risk of cataracts and age-related macular degeneration, which are the main causes of blindness among the elderly (Brown et al., 1999). In addition, lutein and zeaxanthin as well as astaxanthin (which has a similar structure to the first two compounds) exhibited an equally effective antioxidant capacity in rat tracheal epithelial cells, combating the damage caused by the exposure of DNA to ultraviolet A (UVA) radiation and influencing the kinetics of repair. However, adverse effects were observed in human neuroblastoma cells when cultured in the presence of these three carotenoids and subjected to irradiation for a period of 30 minutes; the irradiation caused increased DNA damage, suggesting that the effectiveness of these carotenoids depends on a number of factors, such as their concentration, the cell type, and the time of exposure to radiation as well as the site within the cell and interactions with other antioxidants (Santocono et al., 2006). In an *in vivo* study using rats as an experimental model, a suppressive effect of lutein was observed when administered during the promoter stage of hepatocarcinogenesis at levels that were similar to those observed in humans (Moreno et al., 2007).

Other carotenoids, such as  $\alpha$ -carotene,  $\beta$ -carotene and  $\beta$ -cryptoxanthin, exhibit pro-vitamin A activity.  $\beta$ -carotene has twice the activity of the others carotenoids because it contains two non-replaceable  $\beta$  rings. These carotenoids are cleaved in the intestinal lumen to produce retinol (vitamin A), an essential micronutrient for human health; the World Health Organization (WHO) estimates that there are more than 250 million children worldwide with disabilities related to vitamin A deficiency, with most cases being found in developing countries (<http://www.who.int/nutrition/topics/vad/en/>).

Because of this, biofortification through metabolic engineering has been of great interest, especially with regard to the pro-vitamin A carotenoids. Maize is a target cultivar, as it represents a large fraction of the diet in developing countries. However, to be successful, this strategy requires a controlled increase in the levels of total carotenoids through the increase of biosynthetic pathway flow, minimizing degradation and optimizing kidnapping, in addition to the need to promote a controlled increase of specific compounds, such as  $\beta$ -carotene, which requires extensive knowledge of these metabolic pathways.

In plants, the enzymes involved in carotenoid biosynthesis are encoded in the nucleus and sent to the membranes of chloroplasts, chromoplasts and amyloplasts, where these compounds are synthesized. A simplified metabolic pathway for the biosynthesis of carotenoids (Figure 1) starts with the formation of phytoene from geranylgeranyl pyrophosphate, a step mediated by phytoene synthase (PSY), which as the first enzyme in this pathway, is of vital importance in the synthesis of carotenoids. In maize, there are three known genes encoding this enzyme (*psy1*, *psy2* and *psy3*). Carotene synthesis in the endosperm is predominantly related to the gene *psy1* (Li et al., 2008), and the expression of this gene is correlated with the levels of carotenoids in the endosperm of maize. In white varieties of maize and rice, it was observed that the phenotype is due to a loss-of-function allele of *psy1*, preventing accumulation of carotenoids in the endosperm (Gallagher et al., 2004). In contrast, for the *psy2* gene, high mRNA levels are found in leaves, but this gene seems to have little influence on the level of carotenoids in the endosperm (Palaisa et al., 2003; Gallagher et al., 2004), whereas expression of *psy3* was observed predominantly in roots and embryos, tissues that have limited levels of carotenoids (Li et al., 2008).

Studies show that the *psy1* gene is polymorphic in different varieties of maize (Palaisa et al., 2003). Cloning of the *psy1* gene in maize via transposons resulted in detecting the gene product *Yellow1* (*y1*), which led to increased accumulation of carotenoids in the endosperm. QTL analysis showed that *psy1* and *zds* (zetacaroteno-desaturase) are associated with variations in individual and total carotenoids. Use of the 27 kDa  $\gamma$ -zein promoter or the modified promoter "super  $\gamma$ -zein 27kDa" also increased the accumulation of carotenoids, with a preference for  $\beta$ -carotene in the endosperm (Marks et al., 1985; Marzabal et al., 1998; Wu et al., 2010). A more than 34-fold increase in endosperm carotenoids in white varieties was obtained using the "super-zein promoter" together with overexpression of *crtB* (an *Escherichia coli* gene whose function is related to the *psy* gene) and *crtI* (an *Escherichia coli* gene responsible for four desaturation reactions converting phytoene to lycopene) (Aluru et al., 2008). Moreover, the carotenoid levels and composition in wheat germ and endosperm are dramatically affected by the levels of expression of the *lcy $\beta$*  (lycopene  $\beta$ -cyclase) and *lcy $\epsilon$*  (lycopene  $\epsilon$ -cyclase) genes (Harjes et al., 2008). Fraser and Bramley (2004) showed that *lcy $\epsilon$*  controls the zeaxanthin/lutein ratio and that it is a key gene determining the content of pro-vitamin A in maize. Through analysis of association maps, gene expression analysis and mutagenesis, Harjes et al. (2008) showed that a variation in the gene *locus* for *lcy $\epsilon$*  decreases the flow of the  $\alpha$ -carotene branch, increasing the flow of  $\beta$ -carotene in the metabolic pathway of carotenoids. Four natural polymorphisms in *lcy $\epsilon$*  explained 58% of the variation in these branches and a difference of three times in pro-vitamin A.

Vallabhaneni and Wurtzel (2009) showed that the abundance of mRNA for six genes that encode functional carotene hydroxylase (*hyd*), as well as *cyp97c* gene (encodes  $\beta$ -carotene hydroxylase - P450) and *cyp97a* gene (encodes to  $\epsilon$ -carotene hydroxylase - P450) varied between

different tissues in maize plants and during the development of the endosperm. Natural variability in the gene for  $\beta$ -hydroxylase 1 (*hydb1*) explained 23% of the levels of  $\beta$ -carotene in grain and 37% of the ratio of  $\beta$ -carotene to total carotenoids. A combined analysis of the genes *hydb1* and *lyce* explained 43% and 56% of the variation in the  $\beta$ -carotene and  $\beta$ -carotene/total carotenoid ratio phenotypes, respectively (Yan et al., 2008). Similarly, discovery of the locus for hydroxylase3 (*hyd3*), mapped by QTL analysis, showed three natural alleles in varieties of maize that may explain more than 80% of the variation and the approximately 11-fold difference between  $\beta$ -carotene and  $\beta$ -cryptoxanthin in maize (Vallabhaneni et al., 2009). Recently, the expression of other genes related to the metabolic pathway of carotenoid synthesis was also studied together with grain development in genetically variable maize germplasm, showing that the correlation with the accumulation of carotenoids can be positive or negative depending on the post-pollination time analyzed (Vallabhaneni and Wurtzel, 2009).

Efforts to implement improvements in the levels or composition of carotenoids in maize based on the variability of individual carotenoids in landrace populations through the development of new varieties have led to interesting results. Some of the varieties exhibited levels of zeaxanthin up to  $12 \text{ mg kg}^{-1}$  fresh weight, with total carotenoid levels of approximately  $30 \text{ mg kg}^{-1}$  fresh weight. These varieties are promising candidates for breeding programs aimed at improving  $\beta$ -carotene content, as  $\beta$ -carotene is in the same branch of the metabolic pathway as zeaxanthin synthesis (Fanning et al., 2010). Other studies characterizing maize landrace germplasm have observed high variability in carotenoids, allowing joint selection of some varieties that also exhibit high concentrations of proteins and lipids and phenotypic characteristics such as glassy texture for breeding programs (Berardo et al., 2009).

Specifically targeting the biofortification of pro-vitamin A carotenoids, conventional breeding strategies have allowed selection of cultivars with high levels of carotenoids ( $66.6 \mu\text{g g}^{-1}$  DW) and  $\beta$ -carotene ( $13.6 \mu\text{g g}^{-1}$  DW) (Harjes et al., 2008). Menkir et al. (2008) evaluated different strains of yellow maize adapted to tropical climates and observed large differences in the levels of the main carotenoids (lutein, zeaxanthin,  $\beta$ -carotene,  $\beta$ -cryptoxanthina, and  $\alpha$ -carotene) and significant correlations between carotenoids sharing one of two main branches of the biosynthetic pathway of carotenoids, which would make it possible to increase the levels of multiple carotenoid simultaneously by genetic engineering.

Varieties of sweet maize also showed potential for the development of varieties with high levels of zeaxanthin. The color of these varieties was correlated with the levels of zeaxanthin,  $\beta$ -cryptoxanthin,  $\beta$ -carotene and total carotenoids (Fanning et al., 2010). This finding contrasts with the results obtained by Harjes et al. (2008), who found no correlation of these compounds with the color of maize varieties. In addition, a study conducted at the Embrapa Temperate Agriculture (Brazilian Agricultural Research Corporation) demonstrated differential expression of *psy1* and *lut1* (which encodes the enzyme carotene  $\varepsilon$ -cyclase) (Figure 2) between maize landraces from southern Brazil (Messias et al., 2010).

QTLs have also been studied for genes that encode enzymes involved in the carotenoid metabolic pathway, including phytoene synthase (*psy*), phytoene desaturase (*crtl*), lycopene  $\varepsilon$ -cyclase (*lyce*) and lycopene  $\beta$  cyclase (*lyc $\beta$* ) (Vallabhaneni and Wurtzel, 2009).

Researchers are currently studying the biofortification of multiple nutrients in maize. Recent successes achieved using genetic modification strategies include increases in multivitamins in maize, including an up to 169-fold increase in  $\beta$ -carotene, 6-fold increase in L-

ascorbic acid, and twice as much folate in the endosperm compared to wild-type plants. This maize cultivar was created using transfer of cDNAs encoding the genes *crtI* and *psyI* for the biosynthesis of β-carotene, the dehydroascorbate reductase gene from rice (*dhar*) to increase the synthesis of vitamin C, and the *folE* gene from *E. coli* for the synthesis of folate (Naqvi et al., 2009).

Biofortification strategies using fertilization to increase the content of carotenoids in maize have been widely studied. Additionally, some advances in other crop species have shown promising results. For example, fertilization with  $\text{Ca}^{2+}$  and  $\text{NO}^{3-}$  resulted in increased contents of lycopene and β-carotene in pepper (Flores et al., 2009). Nitrogen fertilization increased the total carotenoids content and the DW of cabbage (Kopsell and Kopsell, 2007), and in both cases, the results varied among cultivars.

Abiotic stress methods indicate that moderate long-term irrigation (15 days prior to harvest) with 5 mM NaCl can increase the nutritional value of romaine lettuce, particularly in relation to the contents of lutein and β-carotene, without losses in productivity or appearance (Kim et al., 2008), though more studies addressing the toxicity of this procedure and its influence on other nutritional compounds are needed. Moderate water stress also resulted in changes in the accumulation of these compounds in broccoli (Cogo et al., 2011). However, there are virtually no data investigating these stressors in maize.

Thus, despite studies indicating maize as a major source of diverse functional compounds involved in secondary metabolism that promote health benefits by acting as antioxidants, biofortification efforts have prioritized increments of pro-vitamin A compounds through metabolic engineering and conventional breeding. Although agronomic interventions employing

fertilizers and hormones have shown promising results, especially in crops susceptible to abiotic stress, such interventions have not been used specifically for the biofortification of carotenoids in maize.

### Vitamins E and C

As shown in Figure 1, geranylgeranyl diphosphate can be converted to phytoene, culminating in the synthesis of carotenoids ( $\alpha$  and  $\beta$  carotenes and xanthophylls), or it can be directed to the metabolic pathway of tocopherol synthesis. Vitamin E is the generic term used to describe a group of eight fat-soluble antioxidants with two different structures: the tocopherols and tocotrienols (Liu, 2007). Vitamin E is generally located mainly in the germ of whole grains and can be extracted with the lipid fraction or lost during the process of oil refining (Cukelj et al., 2010). In this case, the processing of maize is a factor that directly influences levels of vitamin E (Fardet et al., 2008).

In maize, the levels of vitamin E range from 0.3 to 0.7 mg 100 g<sup>-1</sup> for most varieties, with  $\alpha$ -and  $\gamma$ -tocopherols being the only vitamin E compounds found in significant amounts (0.005% and 0.009% of total oil, respectively). There are reports of grain containing a tocopherol concentration of 45 mg kg<sup>-1</sup> DW (Panfili et al., 2004). Varieties of green maize contain from 2.4 to 63.3  $\mu$ g of  $\gamma$ -tocopherol g<sup>-1</sup> DW (Kurilich and Juvik, 1999). The high variability observed in the regulation of the synthesis of carotenoids and tocopherols in maize suggests this species would be a promising target for biofortification.

The antioxidant potential of vitamin E is currently widely accepted, and this activity is responsible for maintaining the integrity of lipid membranes as well as acting in DNA repair and other metabolic processes. Tocopherols and tocotrienols act by donating the hydrogen atom of a

free hydroxyl group to free radicals, resulting in resonance stabilization of vitamin E radicals, thus preventing the oxidation of polyunsaturated lipids in cell membranes (Traber, 2007). *In vivo* studies show that vitamin E improves various parameters related to oxidative stress in animals (Golestani et al., 2006) and humans (Martin et al., 1996).

Another mechanism of vitamin E action is shown in its ability to maintain selenium in a reduced state and to inhibit the formation of nitrosamines, especially at a low pH. In rats pretreated with vitamin E, it was observed that there was increased protection against the effects of N-nitrosodiethylamine, a carcinogen that causes oxidative stress in the liver (Bansal et al., 2005). Another study detected reduction of 8-epi-prostaglandin F2a in the plasma of obese Zucker rats after they received a diet supplemented with vitamin E (Laight et al., 1999). In addition, a study examining rats fed a diet of maize showed that the bioavailability of vitamin E was quite satisfactory (Mitchell et al., 1996).

cDNA encoding homogentisic acid geranylgeranyl transferase (*hggt*), which catalyzes an important step in tocotrienol biosynthesis, was isolated from barley, wheat and rice. Transgenic expression of *hggt* from barley leaves in *Arabidopsis thaliana* resulted in a greater accumulation of tocotrienols. Similarly, induction of *hggt* expression in maize resulted in a more than six-fold increase in tocopherol and tocotrienol contents (Cahoon et al., 2003). These results demonstrate the possibility of increasing the antioxidant content of crops by introducing an enzyme that redirects metabolic flux.

Another compound that represents an appealing target for biofortification in maize is L-ascorbic acid, the precursor of vitamin C. Although cereals are not considered a significant source of ascorbic acid, increasing the levels of this molecule could be an interesting option for

cereals, as they are components of the base of the food pyramid. Although the subcellular site of L-ascorbic acid synthesis has not yet been completely elucidated, this process is known to occur in mitochondria and/or chloroplasts (Ishikawa, 2006). After it is used, L-ascorbic acid can be regenerated to its oxidized form in a reaction catalyzed by the enzyme dehydroascorbate reductase (DHAR). Expression of wheat *dhar* in maize and tobacco resulted in increases in the expression of *dhar* of 32 and 100 times, respectively, as well as 2 to 4 times the levels of ascorbic acid. In addition, the levels of glutathione, the reducing agent used by DHAR, also increased (Chen et al., 2003).

### **Phenolic compounds**

Phenolic compounds are products of secondary metabolism in plants and are derived from the shikimate metabolic pathway, which culminates with the synthesis of phenylalanine and tryptophan. These amino acids are precursors of phenylpropanoid and the flavonoids, isoflavones, pterocarpans, stilbenes, coumarins, fenolamines, aurones, chalcones, lignans and lignins (Treutter, 2010) (Figure 2).

The majority of the phenolic compounds in maize are phenolic acids, such as ferulic, vanillic, caffeic, syringic, synaptic and  $\rho$ -coumaric acids, and polyphenols, such as lignins and lignans (Sosulski et al., 1982). Ferulic acid in whole grains can exist in free, soluble conjugate or complexed forms; the last form is responsible for up to 93% of total ferulic acid. In maize, the levels of these forms exhibit proportions of 0.1:1:100 (Adom and Liu, 2002), constituting up to 2-4% of the shell of the grain measured in dry weight (114), or more than 26-33 g of ferulic acid per kg of maize bran. However, many studies have reported levels of phenolic compounds based on aqueous extraction using methanol, ethanol and acetone (Ortiz-Monasterio et al., 2007; Zhu et

al., 2007; Cakmak, 2008). These methods assume that long extraction times and/or more finely ground samples can maximize the extraction of these compounds from grains. However, these methods extract only soluble phenolics, and more exhaustive extraction techniques, such as digestion, are required for the extraction of complexed phenolics from grains (Liu, 2007).

Phenolic compounds are responsible for essential functions in plant growth and development and as a defense mechanism against pathogens, parasites and predators as well as contributing to the phenotype of plants. Studies also show the action of these compounds in the diets of humans and animals, reducing the risk of developing chronic diseases and cancers (Liu, 2007). However, the mechanisms of action of phenolic compounds *in vivo* have not yet been fully elucidated. These compounds contain one or more aromatic rings with one or more hydroxyl groups (Figure 2), which have antioxidant potential (Rice-Evans et al., 1997). Thus, they are believed to act primarily as free radical scavengers and/or as chelators of transition metals (minerals or trace elements). However, it is unknown whether their ability to trap free radicals is sufficient to explain their antioxidant activity *in vivo* due to their relatively low availability (0.3 - 2.6%) in the digestive tract (Scalbert and Williamson, 2000). It is possible that this concept is a simplification of the mode of action of these compounds. It is more likely that cells respond to polyphenols mainly by direct interactions with receptors or enzymes involved in signal transduction, which may result in modification of the redox status of the cell, generating a series of redox-dependent reactions (Scalbert et al., 2005). Phenolic acids, such as caffeic and ferulic acids, exhibit anti-carcinogenic action, which may involve induction of detoxification systems, especially phase II conjugation reactions (Slavin, 2003).

Other physiological mechanisms of action associated with whole grains are probably connected to the induction/repression of particular genes via cellular signaling through transcription factors, activating antioxidant response elements, which can lead to the synthesis of antioxidant compounds, such as glutathione, or enzymes involved in glutathione metabolism (Myhrstad et al., 2002). Moreover, these phenolic compounds can act as pro-oxidants and can induce apoptosis and, thus, prevent tumor growth (Scalbert et al., 2005).

Another important issue to consider is that only the soluble and free fractions of ferulic and synapic acids, which correspond to limited amounts in cereal, are absorbed in the upper intestinal tract. Nevertheless, most phenols are conjugated and metabolized, resulting in absorption in the colon (Scalbert and Williamson, 2000). For example, ferulic acid is esterified to arabinose residues in the cell wall; this ferulic acid-bound fraction and other polyphenols may be released later in the colon through fermentation, providing beneficial action in a localized form (Adom and Liu, 2002). Thus, whole grain-based foods would provide antioxidant protection for a long period of time along the digestive tract, creating an environment that would protect the intestinal epithelium against pro-oxidative compounds.

Adom and Liu (2002) found the highest levels of phenolic compounds ( $2.12 \text{ mmol g}^{-1}$  grain, 85% in complex form) and total antioxidant activity ( $157 \mu\text{mol g}^{-1}$  grain, 87% in complex form) in maize kernels. However, unlike other cereals, the antioxidant capacity of maize *in vitro* is not significantly correlated with polyphenol content (Fardet et al., 2008), requiring further studies to correlate the antioxidant effects provided by cereals with other types of bioactive compounds. In addition, the antioxidant activity of whole grains is usually underestimated *in vitro*, mainly due to the fraction of polyphenols that are associated with fiber, which in the case

of maize, reaches 87% (Adom and Liu, 2002). Thus, these results are an approximation of the actual antioxidant effect of maize grains *in vivo* due to factors such as differences in the solubility and/or bioavailability of these compounds in the digestive tract and metabolism (Fardet et al., 2008). Although the soluble fraction of phenolic compounds in grains is highly relevant to their activity *in vivo* due to their increased availability in the digestive tract (Gallardo et al., 2006), Pellegrini et al. (2006) showed that in all cases, the complexed fractions of eighteen cereals showed higher antioxidant capacities than free extracts (extracts soluble in organic solvents).

It is known that digestion increases the antioxidant capacity of cereals and their products (Liyana-Pathirana and Shahidi, 2005). Stomach acids and enzymatic hydrolysis in the duodenum increase the solubility and activity of polyphenols in cereals via partial hydrolysis. Thus, *in vitro* digestion using enzyme extracts under conditions simulating the gastrointestinal tract showed that the amount of antioxidants released by the array of cereals in the human gut may be higher than expected based on measurements made with the generally employed aqueous-organic extracts (Perez-Jimenez and Saura-calixto, 2005). Nagah and Seal (2005) also demonstrated the significant influence of *in vitro* gastrointestinal digestion in increasing the release of antioxidants from foods based on whole grains. Similarly, digestion of starch and protein can also increase the release of polyphenols.

An important factor that should be considered within the context of biofortification is that processing maize can also change the content of phenolic compounds. Dewanto et al. (2002) performed thermal processing at 115° C for 25 min for the purpose of canning common yellow maize and noticed increases of 44% in antioxidant activity, 550% in the content of ferulic acid

and 54% in the total phenolic content compared to fresh maize, despite the loss of 25% of the vitamin C content. Increases in the concentration of total phenolics and antioxidant activity were also reported by Randhir et al. (2007) during thermal sterilization of maize. Inhibition of the bacterium *H. pylori*, which is associated with ulcer occurrence, was also noted. According to the authors, these changes in functionality due to heat processing may be due to changes in the content and profile of total phenolic compounds induced by oxidation or polymerization.

Maize, like other cereals, represents a good source of polyphenols. Most of these compounds are located in the endosperm of the grain. Lignans and lignins are predominant in the cell wall. Lignin polymers, which are highly branched polyphenolic compounds (Figure 2), constitute 30% of the biomass of plants and belong to the most abundant class of polymers on the planet. Lignins represent a major component of whole grains, accounting for 3-7% of the bran fraction, and these compounds were long considered to be nutritionally inert in the digestive tract. However, their polyphenolic structure gives them antioxidant potential (Fardet et al., 2008).

Lignans are secondary constituents of plants belonging to the group of phytoestrogens. The lignans includes lariciresinol, syringaresinol and pinoresinol as well as secoisolariciresinol and matairesinol (Figure 2). The last two substances are the major lignans found in plant foods, especially cereals such as maize, oats, wheat and rye (Thompson et al., 1991). Both matairesinol and secoisolariciresinol are converted to enterodiol and enterolactone (mammalian lignan) by the intestinal microflora. It is possible that the loss of the methoxy radical during digestion results in the reduced antioxidant capacity observed between the two groups (Niemeyer and Metzler, 2001). The antioxidant activity of these plant lignans has been observed in different model

systems, both aqueous and lipid, showing a reduction of lipid oxidation (Kitts et al., 1999), although their effects are less pronounced than lignin in the prevention of genetic oxidative damage, as shown in human colon cells incubated with enterolactone (Pool-Zobel, 2000). Begum et al. (2004) showed that rats can metabolize lignans into lignins. In another study, diabetic rats (diabetic nephropathy) injected subcutaneously with nordihydroguaiaretic acid (an antioxidant and lignin lipoxygenase inhibitor) for four weeks showed less renal dysfunction and oxidative stress than control (Anjaneyulu and Chopra, 2004).

Although studies have shown that lignins and lignans have antioxidant effects *in vivo*, particularly in the colon, few studies have been conducted on their bioavailability in the intestine. The majority of studies addressing the antioxidant potential of these compounds have been performed *in vitro* using mammals cells, mainly focused on their influence on DNA damage (oxidative damage) (Fardet et al., 2008; Cooke et al., 2002).

Searching for increases in the levels of phenolic compounds in plants, several studies have been conducted on the metabolic pathway involved in the production of these compounds. The key enzymes associated with this route are chalcone synthase (CHS) and isoflavone synthase (IFS), known as cytochrome P450. From these enzymes, the structural diversity of flavonoids is derived via substitution of the carbon basic skeleton through hydroxylation, glycosylation, methylation, acylation, prenylation and polymerization (Dixon and Pasinetti, 2010). However, the enzymes that catalyze substitution reactions are often encoded by large gene families with conserved sequence motifs, which have hampered attempts at improvement of phenolic compounds by genetic modification. In addition, many of these enzymes are active with multiple classes of flavonoids (Peel et al., 2009). The subcellular localization of the components

associated with the precursors of phenylalanine also remains uncertain (Zhao et al., 2010), and little is known about these components are deployed out of the vacuole (Gomez et al., 2009).

Thus, despite the interest in increasing the contents of phenolic compounds in plants, reports in the literature regarding maize in which the phenol content was genetically modified are scarce. Studies on the use of transcription factors for induction of multiple genes in this metabolic pathway have generated the most promising results (Grotewold et al., 1998; Grotewold et al., 2000; Rhee et al., 2010; Hichri et al., 2011). Most of the structural genes encoding enzymes involved in the biosynthesis of anthocyanins appear to be coordinately regulated by bHLH transcription factors, including B and R, interacting with the MYB gene *c1* (Chandler et al., 1989). Ectopic expression of R and C1 in an *in vitro* culture of non-pigmented maize cells resulted in biosynthesis and accumulation of anthocyanins due to the coordinated expression of most structural genes (Grotewold et al., 2000). Expression of P (a MYB-type transcriptional regulator) in maize cells also induced the expression of a coordinated series of biosynthetic genes leading to the accumulation of flavonoids different than those regulated by C1/R (Grotewold et al., 2000). Additionally, Dias and Grotewold (2003) used a maize cell culture system to investigate the consequences of the accumulation of metabolites by expressing the R2R3 MYB transcription factor (ZmMyb-IF35) and found that ZmMyb-IF35 does not induce accumulation of flavonoids, but does induce the accumulation of ferulic and chlorogenic acids. In soybean seeds, genes involved in the phenylpropanoid metabolic pathway were activated by the expression of transcription factors C1 and R from maize, resulting in a slight increase in the levels of isoflavones. On the other hand, co-suppression of flavonone 3-hydroxylase to block the synthesis of the anthocyanin arm of this metabolic pathway together with the expression of C1/R

resulted in high levels of isoflavones, which may represent a promising strategy for the biofortification of flavonoids in cereals (Yu et al., 2003).

With the same purpose, a number of studies have assessed the diversity of maize landraces searching for varieties with higher concentrations of phenolic compounds. Del Pozo-Insfran et al. (2006) demonstrated that one variety of Mexican purple maize showed a significantly higher antioxidant capacity than American purple and white varieties, which was attributed to the specific anthocyanins and/or the composition of polyphenols in the plants. Lopez-Martinez et al. (2008) evaluated 18 varieties of native Mexican maize and observed a varied concentration of phenolic compounds, anthocyanins and ferulic acid, identifying three varieties, all purple, with the highest content of these phytochemicals. Likewise, Pedreschi and Cisneros-Zevallos (2007) observed high levels of anthocyanins and non-anthocyanic phenolic compounds in a variety of purple maize from a region of the Peruvian Andes. In maize hybrids, equal variability was observed in the levels of phenolic compounds, with moderate digestibility in the small intestine of pigs (Kljak et al., 2009).

Mineral fertilization has also been used to increase the contents of some phenolic compounds, as maintenance of mineral levels is a prerequisite to provide cofactors for many enzymes in the phenylpropanoid pathway. For example, magnesium ( $Mg^{2+}$ ) and manganese ( $Mn^{2+}$ ) ions participate in the activity of phenylalanine ammonia lyase (PAL), CoA-ligases, and methyltransferases (Treutter, 2010). On the other hand,  $Ca^{2+}$  deficiency induced the accumulation of total phenolics in *Prunus callus* (Yuri et al., 1990). Phosphate deficiency, in turn, increased the accumulation of chlorogenic acid in *Helianthus annuus* (Koeppel et al., 1976) and flavanones in tomatoes (Zornoza and Esteban, 1984). Boron (B) deficiency also led to the

accumulation of phenolic compounds (Shkolnik, 1984) in an *in vivo* grape callus system, and the increases in B content caused a decline in catechins and proanthocyanins, while addition of AlCl<sub>3</sub> increased the content of flavonoids, possibly due to stress caused by aluminum (Feucht et al., 1999). The accumulation of phenolic compounds is often affected by high levels of N, as described for oats (Norbaek et al., 2003), apricot fruit (Radi et al., 2003), *Vaccinium myrtillus* (Wetzell and Shevtsova, 2004), *Pinus elliotii* (Saxon et al., 2004), apple (Lesser and Treutter, 2005) and recently *Tobacco* (Treutter, 2010). In strawberries, it appears that excessive fertilization with N promotes the accumulation of large amounts of phenolic compounds. However, conflicting results were reported by Anttonen et al. (2006), who showed a reduction in the concentration of phenolics in strawberry plants after increased fertilization with N. In *Solanum carolinense*, an increase in phenolics was observed to be associated with an increasing demand for N. Surprisingly, in wheat (*Triticum aestivum*), an increase in soluble phenolic acids in straw but a decrease in grain occurred when N demand was high (Treutter, 2010).

An increase in levels of phenolic compounds can also be obtained through addition of Se. Basil plants treated with 10-50 mg of Se dm<sup>-3</sup> and 3-20 mg of Se dm<sup>-3</sup> exhibited increased levels of anthocyanins and total phenolics, respectively (Hawrylak-Nowak, 2008). Fertilization of broccoli with S influenced the content of flavonoids and hydroxycinnamic acid derivatives (ferulic acid and sinapism acid derivatives + caffeoyl-quinic acid derivatives) (Vallejo et al., 2003).

Thus, the literature shows that improvement of antioxidant phenolic content can be achieved in maize, whose studies are attempted especially in the use of fertilizers. Combining this strategy with the increasing knowledge of the mechanisms regulating the synthesis of these

compounds as well as exploitation of the genetic variability of maize landraces should lead to promising results regarding the biofortification of phenolic compounds in maize.

## Perspectives

Maize, in addition to being the main staple food of the population in many countries, is used in the production of hundreds of products. Its importance in human nutrition and animal feed and, hence, throughout the food chain is undeniable, making it a target species in current attempts at biofortification. In this context, Table 1 summarizes the main strategies and advances presented in this article related to biofortification of micronutrients, with focus in maize. From the studies cited here, it is clear that maize is considered a good source of functional antioxidant compounds containing high concentrations of xanthophylls, especially lutein and zeaxanthin, related to the prevention of retinal diseases, as well as phenolic compounds (especially ferulic acid) and several other compounds, including lignins and lignans, all of which play important roles in the antioxidant defense system of the body and have thus far been largely neglected. Maize also exhibits considerable levels of important trace minerals, such as selenium. However, deficiencies of carotenoids, such as precursors of vitamin A, and the lack of availability of some nutritional compounds, such as iron and zinc, in maize have also been extensively studied for the purpose of further biofortifying this crop.

Exogenous supplementation and fortification, particularly of minerals and vitamins, have been extensively used in many countries; however, these approaches are expensive and difficult to carry out, especially in developing countries. Fortification based on endogenous fertilization techniques has been shown to be more economically viable and quite effective. Nevertheless, despite the large number of studies addressing processes involved in improving food quality,

such as genes and metabolisms, the influence of fertilization on these parameters and the mechanisms through which they occur remain uncertain and controversial. Furthermore, these effects are dependent on the cultivar, the doses applied and the source of fertilizer used (Zhao et al., 2004). Another bottleneck of these agronomic interventions is the environmental cost and impact of these fertilizers, as great amounts of these substances are leached, eventually contaminating the soil and groundwater (Sors et al., 2005). Therefore, investigations of alternative and natural sources of fertilizers as well as application methods to address the gradual impoverishment of the soil, maximizing plant uptake and preventing contamination, are required (Cakmak, 2008).

Likewise, the genetic variability related to the nutrient concentrations in different maize germplasm has led to the extensive exploration of conventional breeding, despite the fact that this is a long-term strategy. The use of transgenic techniques has also been widely studied, with promising results being obtained, but these studies often lack correlation of the results with data on technical and economic feasibility and the influence on productivity parameters, making it difficult to assess the real gains obtained. In addition, interaction with other quality parameters associated with these products due to the multiple interrelationships that exist between metabolism pathways becomes difficult to assess.

However, the rapid advancement of new approaches in biochemistry and molecular biology using techniques such as transcriptomics and metabolomics presents the possibility of new studies addressing the mechanisms that regulate the synthesis of functional and nutritional compounds. These new techniques make it possible to investigate ways to modify metabolism by either increasing the contents of these compounds in maize or increasing their bioavailability as

well as allowing the verification of different metabolic pathways affected because of the improvement of these compounds (Treutter, 2010). In this context, a combination of different techniques is probably the biofortification strategy that will generate the best results.

Considering the present and future focus on the use of plant species for the production of biofuel from biomass, therapeutic and industrial products as well as for the production of food with improved nutrition and functional value, maize represents a very promising target crop species. From the perspective of the consumer, a focus on increased nutrients and functional compounds that provide improved nutrition and health is of great interest. Development of maize plants with improved characteristics will involve overcoming a number of challenges, both technical and regulatory, and especially issues related to the complex changes that may occur as a function of the manipulation of plant metabolism.

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**Table 1.** Summary of the main strategies and advances presented in this article related to the biofortification of maize grains.

Micronutrient	Biofortification strategy	Results	Reference
Minerals	Conventional breeding and assisted selection	New varieties with high concentrations of Zn and Fe	Gregorio (2002) Cakmak (2008) Simic et al. (2009)
		Characterization of SSR and SNP markers associated with transport of Zn and Fe	Sharma and Chauha (2008)
	Genetic transformation	Overexpression of Zn/Fe transporter protein increases its levels in plants but also increases toxic elements such as Cd	Ramesh et al. (2004) Palmgren et al. (2008)
		The NAC transcriptional factor increases Zn and Fe in grain	Uauy et al. (2006)
		Overexpression of the YS1 gene, coding for nicotianamine aminotransferase, increases Fe accumulation	Curie et al. (2001)
		Overexpression of the phytase gene ( <i>Phya</i> ) and increasing ferritin protein results in higher levels and bioavailability of Fe	Drakakaki et al. (2005)

		Increases in secondary transporters of $\text{Ca}^{+2}/\text{H}^{+}$ promotes higher $\text{Ca}^{+}$ accumulation	Park et al. (2005, 2009)
		Application of NPK with Zn lignosulfate increases Zn levels	Martin-Ortiz et al. (2003)
		Foliar application of Zn in wheat increases Zn levels in Zn-poor soils	Cakmak (2008)
		Equilibrium between N, P and/or K increases absorption of Fe, Zn, Ca, Cu, P, K, Mg, S and Mn	Frossard et al. (2000)
Fertilizers or inductors		Application of N increases Fe, K, P, Ca, Mg, S, Cu, Fe, Mn and Zn	Zang et al. (2008) Farcora et al. (2001)
		Application of sodium selenate increases accumulation and bioavailability in grain	Broadley et al. (2006) Fang et al. (2008) Li et al. (2010)
		Supplying Se in NPK increases Se levels in grains	Combs (2001) Gomez-Galera et al. (2010)
Carotenoids	Conventional breeding, assisted selection and mutagenesis	New varieties with high zeaxanthin and $\beta$ -carotene contents	Fanning et al. (2010) Berardo et al. (2009) Harjes et al. (2008) Menkir et al. (2008) Messias et al. (2010)
		Varieties with higher <i>psyI</i> and <i>zd2</i> expression accumulate high carotenoid contents	Palaisa et al. (2003) Marzabal et al. (1998) Wu et al. (2010)

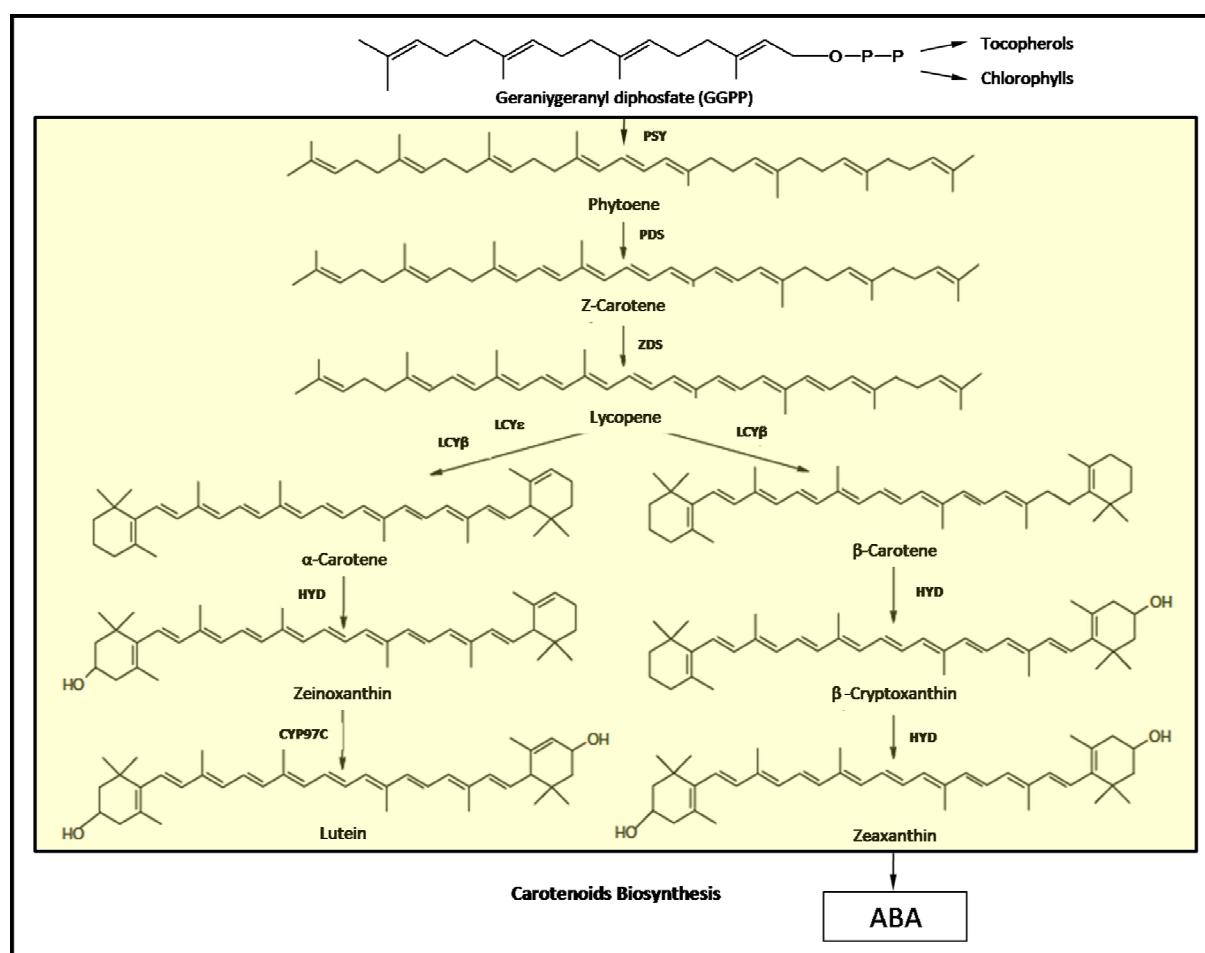
Genetic transformation	Overexpression of <i>crtI</i> and <i>psyI</i> (biosynthesis of β-carotene), <i>dhar</i> (synthesis of ascorbic acid) and <i>folE</i> (synthesis of folate) increased these metabolites in maize	Naqvi et al. (2009)
	Overexpression of the <i>crtB</i> and <i>crtI E. coli</i> genes increases carotenoid accumulation in white maize varieties	Aluru et al. (2008)
Fertilizers	Application of Ca <sup>+</sup> and NO <sup>3-</sup> increases lycopene and β-carotene synthesis in pepper	Flores et al. (2009)
	Nitrogen fertilization increases the total carotenoid content in cabbage	Kopsell and Kopsell (2007)
Abiotic stress	Moderate water and salt stresses can stimulate carotenoid synthesis	Kim et al. (2008) Cogo et al. (2011)
	Varieties with high vitamin E and tocopherols	Panfili et al. (2004) Kurilich and Juvik (1999)
Vitamin C and E	Overexpression of the <i>hggt</i> gene resulted in increased tocopherol and tocotrienol contents	Cahoon et al. (2003)
	Overexpression of the <i>dhar</i> gene resulted in increased L-ascorbic acid content	Chen et al. (2003) Naqvi et al. (2009)

Phenolic compounds (PC)	Conventional breeding	Varieties of maize with high PC contents	Adom and Liu (2002) Del Pozo-Insfran et al. (2006) Lopez-Martinez et al. (2008) Pedreschi and Cisneros-Zevallos (2007) Kljak et al. (2009)
		<i>In vitro</i> ectopic expression of a bHLH transcription factor that interacts with the MyB gene C1 increases anthocyanin synthesis	Chandler et al. (1989) Grotewold et al. (1998, 2000)
		<i>In vitro</i> ectopic expression of the P transcription factor increases flavonoid accumulation	Grotewold et al. (2000)
		<i>In vitro</i> ectopic expression of the R2R3 MYB transcription factor (ZmMyb-IF35) increases ferulic and chlorogenic acid accumulation	Dias and Grotewold (2003)
		Co-suppression of flavonone 3-hydroxylase and the C1/R transcription factor blocks anthocyanin synthesis and stimulates isoflavone accumulation	Yu et al. (2003)
Fertilizers	Mg <sup>+2</sup> and Mn <sup>+2</sup> stimulates phal, CoA ligase and methyltransferase activity	Treutter (2010)	
	Deficiency of Ca <sup>+2</sup> and B induces accumulation of PC	Yuri et al. (1990) Shkalnik et al. (1984)	

	Phosphate deficiency increases the accumulation of chlorogenic acid and flavonones	Koeppe et al. (1976) Zornoza and Esteban (1984)
		Norbaek et al. (2003)
		Radi et al. (2003)
		Saxon et al. (2004)
	Supplying N stimulates accumulation of PC	Wetzell and Shevtsova (2004)
		Lesser and Treutter (2005)
		Treutter (2010)
	Supplying N reduces accumulation of PC	Anttonen et al. (2006)
	Supplying Se increases anthocyanins and total phenolics	Hawrylak-Nowak (2008)
Abiotic stress	Moderate abiotic stress caused by AlCl <sub>3</sub> induces PC accumulation	Feucht et al. (1999) Cogo et al. (2011)

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**Figure 1.** Metabolic pathway of carotenoids and some of the key enzymes involved in this metabolism, including phytoene desaturase (PDS),  $\zeta$ -carotene desaturase (ZDS), lycopene  $\epsilon$ -cyclase (*lycε*), lycopene  $\beta$ -cyclase (*lycβ*),  $\epsilon$ -carotene hydroxylase (CYP97C) and  $\beta$ -carotene hydroxylase (HYD). ABA: abscisic acid synthesis.



**Figure 2.** Metabolic pathway of the biosynthesis of the main phenolic compounds found in maize. The two lignans presented exemplify the major lignans found in cereals. Dotted arrows indicate suppression of steps in the process of biosynthesis.

