1

Strategies for Iron Biofortification of Crop Plants

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1. Introduction

Iron (Fe) is an essential element for all living organisms because of its property of being able to catalyze oxidation/reduction reactions. Fe serves as a prosthetic group in proteins to which it is associated either directly or through a heme or an iron-sulfur cluster. It exists in two redox states, the reduced ferrous Fe^{2+} and the oxidized Fe^{3+} form and is able to loose or gain an electron, respectively, within metalloproteins (e.g. Fe-S cluster or heme-Fe proteins). Such metalloproteins are involved in fundamental biochemical reactions like the electron transfer chains of respiration and photosynthesis, the biosynthesis of DNA, lipids and other metabolites, the detoxification of reactive oxygen species.

The cellular processes that involve Fe take place in distinct intracellular compartments like e.g. cytoplasm, mitochondria, plastids, cell walls, which therefore need to be provided with an adequate amount of Fe. Since this metal is involved in a wide range of essential processes, the undersupply with Fe leads to severe deficiency symptoms in the affected organism.

Fe deficiency is one of the most prevalent and most serious nutrient deficiencies threatening human health in the world, affecting approximately two billion people (de Benoist et al., 2008). Various physiological diseases, such as anaemia and some neurodegenerative diseases are triggered by Fe deficiency (Sheftela et al., 2011). Especially those countries are affected by Fe deficiency diseases, where people have low meat intake and the diets are mostly based on staple crops. Young children, pregnant and postpartum women are the most commonly and severely affected population groups, because of the high Fe demands of infant growth and pregnancy (de Benoist et al., 2008). Human health problems caused by Fe deficiency can be prevented by specific attention to food composition and by choosing a balanced diet with sufficient and bio-available Fe content.

Several possibilities exist to enrich the diet with bio-available Fe, which all have advantages and disadvantages. Supplementation of Fe in the diet is possible by supply of Fe chelates and salts in form of pills (Yakoob & Bhutta, 2011). However, formulations which are well tolerated by patients are expensive and particularly in underdeveloped areas of the world difficult to supply daily, as additional systems for purchasing, transport and distribution

have to be established, associated with extra costs. The fortification of food products like flour with Fe salts is also effective (Best et al., 2011) and in place in some developed countries (Huma et al., 2007). Generally, an existing food industry is required for food processing, so that again supply is difficult in underdeveloped countries. The diversification of the diet with an emphasis on improvement of Fe-rich food crops like certain green leafy vegetables and legume seeds would be highly effective and desirable. In fact, it is actually the simplification of the diet with its low diversification that is the main cause of the micronutrient deficiency (Nair & Iyengar, 2009). The structure of agriculture, the green revolution and the need to supply sufficient food in light of a rapidly increasing world population had caused a concentration on calorie-rich carbohydrate-providing crops (Gopalan, 1996). Finally, the bio-fortification of staple crops is considered to be a very effective method which would reach many people even in underdeveloped countries (Bouis et al., 2011). A prerequisite is that the local staple crops are bio-fortified so that they contain more and better available Fe. This can generally be reached by breeding, which is performed either by the breeding industry or by governmental agencies. The newly bred lines need to be distributed to and accepted by the local farmers. In any case, it seems that the prevention of Fe deficiency in the population of underdeveloped countries may strongly depend on governmental willingness, administration and regulation concerning the quality and quantity of food. It is clear that none of the above mentioned treatments is "cheap". Yet, the economic losses due to fatigue and neuronal dysfunctions might be far greater in negative value than the expected expenses to prevent these problems (Hunt, 2002). Therefore, the combat against Fe deficiency diseases is among the top priorities particularly listed by the WHO (de Benoist et al., 2008).

Here, we present some of the approaches for bio-fortification of crops with Fe. This report will focus on the underlying technological advances and our knowledge about the physiological processes leading to the enrichment of specific plant organs with Fe and their increased bio-availability.

2. Overview about Fe homeostasis in plants

The most important plants for nutrition of humans and mammals are the highly evolved flowering plants (angiosperms). These include the major crops and plant model organisms like rice, maize, legumes and *Arabidopsis thaliana*. Fe is found in all plant organs, which include roots, leaves, flowers, fruits with seeds, storage organs like tubers. Depending on the plant crop species and its use all these various parts can be edible, and in this case the concentrations of bio-available Fe should be high. Under natural conditions, all Fe of living organisms ultimately enters the nutrition chain via plant roots. In the soil, Fe mainly exists as Fe^{3+}, often bound as iron hydroxides in mineral soil particles (Marschner, 1995). Plants need a Fe concentration of 10^{-6} M for optimal growth, but the concentration of free Fe^{3+} in an aerobic, aqueous environment of the soil with a pH of 7 is about 10^{-17} M. At lower pH the solubility of Fe is increased, but a Fe^{3+} concentration of 10^{-6} M is reached at pH 3,3 (Hell & Stephan, 2003). 30% of the world`s crop land is too alkaline for optimal plant growth. Moreover, it appears that some staple crops, like rice, are especially susceptible to Fe deficiency. Under alkaline and calcareous soil conditions, bioavailable Fe concentrations are low in the soil despite of the abundance of this metal in the earth crust. To meet their demand for Fe, plants need to mobilize Fe in the soil by rendering it more soluble before

they are able to take it up into their roots. Two effective Fe acquisition systems known as Strategy I and Strategy II have evolved in higher plants, based on reduction and chelation of Fe^{3+}, respectively (Römheld, 1987; Römheld & Marschner, 1986). The group of strategy I plants includes all dicotyledonous and all non-grass monocotyledonous plants. They acidify the soil, reduce Fe^{3+} and take up divalent Fe^{2+} via specific divalent metal transporters (Jeong & Guerinot, 2009; Morrissey & Guerinot, 2009). All monocotyledonous grasses are Strategy II plants, including all major cereal crop plants like rice (*Oryza sativa*), barley (*Hordum vulgare*), wheat (*Triticum aestivum*) and maize (*Zea mays*). These plants synthesize and secrete Fe^{3+}-chelating methionine derivatives termed phytosiderophores of the mugineic acid family and subsequently take up Fe^{3+}-phytosiderophore complexes (Jeong & Guerinot, 2009; Kobayashi et al., 2010; Morrissey & Guerinot, 2009). Fe reaches leaves mainly in complexed form with citrate through the xylem, which is a plant conductive tissue for water and mineral long-distance transport. Typical sink organs like immature organs receive Fe via the phloem pathway, which represents the conductive tissue for assimilates and signals. Inside plants, Fe is distributed to all tissues and cellular compartments through the activities of several different types of membrane-bound metal transport proteins (Curie et al., 2009; Jeong & Guerinot, 2009). Metal ions are predominant in a bound or chelated form inside cells to enhance solubility and transport but at the same time minimize toxicity effects of metal ions. In plants, oganic acids like citrate and malate, the amino acid histidine and the plant-specific methionine derivative nicotianamine are mainly involved in Fe transport and solubility (Briat et al., 2007; Callahan et al., 2006). Chelators for metals also include polypeptides such as phytochelatins (PCs) and metallothioneins (MTs) which are essentially involved in the tolerance to potentially toxic heavy metal ions (Hassinen et al., 2011; Pal & Rai, 2010). Fe can be stored in form of ferritin in the plastids which also serves to reduce oxidative stress (Briat et al., 2010b). In the vacuole Fe is frequently bound by phytic acid, which is composed of inositol esterified with phosphorous acid. The ionized form binds several mineral ions including Fe. It is present in cereal grains, nuts and leguminous seeds (Gibson et al., 2010).

In conclusion, plants contain a complex regulation network of genes which provide uptake, chelation, transport, sub-cellular distribution and the storage of Fe. Knowing these processes is the prerequisite for their manipulation in order to breed in the future high-quality nutritious crops.

3. Biofortification strategies

Bio-fortification designates the natural enrichment of plants with nutrients and health-promoting factors during their growth. Bio-fortification focuses on generating and breeding major staple food crops that would produce edible products enriched in bioavailable amounts of micronutrients, provitamin A carotenoids or several other known components that enhance nutrient use efficiency and are beneficial to human health (Hirschi, 2009).

The bio-fortification approach is interesting for staple crops that were mainly bred for carbohydrate content, processing characteristics and yield in the past decades, e.g. maize, wheat, rice and also some of the local plants like Cassava, potato and sweet potato. Elite lines highly performing in the field might on the other hand be poor in micronutrient contents (White & Broadley, 2009). Plants with a higher nutritional value can be produced

by classical breeding. In this case, wild relatives or varieties with beneficial micronutrient content are selected and the respective trait crossed into the elite lines. This approach is labor-intensive, it can be aided by the usage of molecular markers that are closely linked with the traits of interest; in an optimal case, the molecular nature of the trait is known and can be followed directly with molecular PCR and sequencing technologies in the various breeding steps (Tester & Langridge, 2010; Welch & Graham, 2004). Alternatively, bio-fortified crops with new properties can be generated using gene technology in addition to classical breeding. In this case, the trait of interest is constructed *in vitro* using molecular cloning to combine promoters and genes that together confer the trait. These constructs are transferred into the crops, which could be achieved for example by biolistic methods based on the bombardment of plant cells with the DNA or using as tool *Agrobacterium tumefaciens*. The integration event of the DNA fragment conferring the new trait into the plant genome is selected, respective transgenic plants are generated and multiplied (Sayre et al., 2011; Shewry et al., 2008). Research on bio-fortification via classical breeding and/or gene technology-based breeding was stimulated by non-profit funding organizations, such as through the program HarvestPlus (http://www.harvestplus.org) (Bouis et al., 2011) and the Golden rice project (http://www.goldenrice.org) (Beyer, 2010). Bio-fortification thus became an agricultural and breeding tool to combat human malnutrition in the world.

For the Fe bio-fortification breeding, several challenges have to be overcome which can be mastered if scientists acquire a better understanding of the physiological mechanisms of plant metal homeostasis and political regulations allow for distributing such modified plants (Hotz & McClafferty, 2007). First, the plants have to increase Fe uptake. Depending on the soil properties, specific strategies for Fe mobilization in the soil have to be employed by the plants. Plants are then able to render Fe in the soil more soluble and bio-available to them. Second, Fe should accumulate in the edible parts of the plant such as seeds and fruits. These plant parts should act as effective sinks for Fe. Third, the nutrients should be preferentially stored in a form that renders them bioavailable for the human digestive system. Fe can be complexed with soluble organic ligands which would increase its bio-availability. However, some compounds like phytic acid can precipitate Fe and act as antinutrients if phytase is not provided.

First attempts to target physiological processes of Fe homeostasis have already been started to test the effect on bio-fortification. Moreover, assays are available to test for uptake of Fe from plant food items (Glahn et al., 2002; Lee et al., 2009; Maurer et al., 2010).

4. Examples for Fe biofortification research in plants

4.1 Reduction of phytic acid content

A successful approach for Fe bio-fortification relies on the reduction of Fe complex-forming metabolites that act as anti-nutrients, like tannins, a phenolic polymer, and phytic acid (Welch & Graham, 2004). Phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate; InsP6) comprises up to 80 % of the total seed phosphorus content and its dry mass may account for 1-2 % of seed weight (Hurrell, 2002). It accumulates as a phosphorous and mineral storage compound in globoids in the seeds of many staple crops, including legumes like soybean, cereal embryo and/or aleurone cells (Bohn et al., 2008). In developing countries, the prevalence of phytic acid in the plant-based diet is believed to contribute to the high rate of

Fe deficiency and anemia. On the other hand, reduction of phytic acid contents is also seen negative, since in a well-balanced diet it has health-promoting effects on the immune system and in preventing kidney stones (Shamsuddin, 2008). Phytic acid content can be reduced by disruption of its biosynthetic chain which would result in a "low phytic acid" (*lpa*) phenotype (Raboy, 2007; Rasmussen et al., 2010). Phytic acid is mainly synthesized from d-glucose-6-phosphate transformed first into 1d-*myo*-inositol-3-phosphate [Ins(3)P_1] (Loewus & Murthy, 2000). Several biochemical pathways seem to be involved in transforming Ins(3)P_1 to InsP_6 in plants, depending on the plant species (Bohn et al., 2008; Rasmussen et al., 2010). Furthermore, an ABC transporter is required for transport and compartmentalization in the final steps which can also be disrupted (Shi et al., 2007). Several mutant lines have been identified in various plant species including soybean (Hitz et al., 2002; Wilcox et al., 2000), maize (Pilu et al., 2003; Raboy et al., 2000), wheat (Guttie*ri et al.*, 2004), rice (Larson et al., 2000; Liu et al., 2007) and Arabidopsis (Kim & Tai, 2011; Stevenson-Paulik et al., 2005). However, conventional breeding may result in strong phytic acid reduction and thereby in counteracting effects of such *lpa* mutants, like decreased germination and reduced seedling growth, if the effect takes place overall in the plants. Better mutants can be created using gene technology since only the late functions of the genes for phytate synthesis may be abolished and only in certain phases and organs during the life cycle of the plants by using specific promoters that allow expression of the transgenes under very controlled conditions (Kuwano et al., 2009; Kuwano et al., 2006).

Alternatively, the late stages of phytic acid biosynthesis and transport may be specifically targeted in mutants (Stevenson-Paulik et al., 2005). For example, two Arabidopsis genes for inositol polyphosphate kinases, ATIPK1 and ATIPK2, have been disrupted, which are required for the later steps of phytic acid synthesis. These mutants were found to produce 93 % less phytic acid in seeds, while seed yield and germination were not affected. It was however found that the loss of phytic acid precursors altered phosphate sensing.

An alternative approach may rely on the transformation of plants with phytase enzymes. Such enzymes are isolated from a multitude of different microorganisms, and heat-stability besides enzyme activity are important criteria to consider in the food processing procedure (Bohn et al., 2008; Rao et al., 2009).

Numerous examinations have to follow to find a solution to exclude negative influences of phytic acid as an anti-nutrient but sustain its positive effects on plant growth. It has to be investigated in future studies how useful phytate-reduced crops are for human Fe uptake.

4.2 Increase of ferritin content

Ferritins are multiprotein complexes consisting of ferritin peptide chains that are organized in globular manner to contain inside up to 4000 Fe^{3+} ions. Existing reports suggest that Fe is stored short- and long-term in ferritins and utilized for the accumulation of Fe-containing proteins. This way, ferritins supply Fe during developmental processes of plants, and some plant species contain high ferritin-Fe levels in seeds (Briat et al., 2010a). Ferritins also serve to alleviate oxidative stress (Briat et al., 2010b). However, not in every case high ferritin levels need to colocalize with high Fe levels in seeds (Cvitanich *et al.*, 2010). Ferritin-Fe is separated from other Fe-binding components by its protein coat and its localization inside plastids or mitochondria. Ferritins exist in all organisms as a store of Fe. Ferritins in general

and ferritins in plant food items provide a high Fe bioavailability (Murray-Kolb et al., 2002; San Martin et al., 2008; Theil, 2004).

Ferritin genes were used in bio-fortification approaches. For example, leguminous ferritin genes, especially from soybean and bean, were over-expressed in plants, and subsequently an accumulation of ferritin protein was observed in the plants. Ferritins from legumes had been used since this plant family contains high ferritin levels in seeds, and the legume seeds serve in human and animal nutrition. Over-expression of ferritins in seeds and cereal grains resulted in an increased Fe content in these edible parts (Goto et al., 1999; Lucca et al., 2002). However, over-expression in vegetative tissues did not have this effect (Drakakaki et al., 2000), and in some cases even caused Fe deficiency symptoms (Van Wuytswinkel et al., 1999). Overall, ferritin over-expression has to studied in more detail and it may be needed to increase Fe uptake at the same time to have a full effect of Fe increases (Qu le et al., 2005).

Thus, research on the influence of ferritin on Fe accumulation and bio-availability as well as its effect on human Fe uptake revealed that this protein is a promising candidate for bio-fortification approaches if utilized in an appropriate manner in plants.

4.3 Increase of nicotianamine content

Nicotianamine is a key compound of metal homeostasis in plants. Nicotianamine is a non-proteinogenic amino acid derived from S-adenosyl methionine by the action of the enzyme nicotianamine synthase. Nicotianamine is able to bind a number of different metals including ferrous and ferric Fe, depending on the pH environment. Nicotianamine ensures that Fe remains soluble inside the cells. Thus, Fe can be transported to the multiple compartments, and Fe toxicity effects are reduced. Nicotianamine contributes to all important sub-processes of plant metal homeostasis: Mobilization and uptake, intercellular- and intracellular transport, sequestration, storage and detoxification of metals. Several studies presented positive effects of nicotianamine on Fe uptake and accumulation in seeds (Cheng et al., 2007; Douchkov et al., 2005; Douchkov et al., 2001; Klatte et al., 2009). Therefore, nicotianamine can be considered to be a potential bio-fortification factor for Fe in seeds and grains of crop plants. (Lee et al., 2009) showed that overexpression of a nicotianamine synthase gene, *OsNAS3*, resulted in an increase of Fe in leaves and seeds, and that in seeds a higher nicotianamine-Fe content was present. Moreover, it was found that these transgenic seeds provided a better source of dietary Fe than the wild type seeds (Lee et al., 2009). (Zheng et al., 2010) demonstrated by seed-specific expression of OsNAS1 that rice grains contained a higher amount of nicotianamine. These transgenic rice grains performed better in Fe utilization studies using human cells (Zheng et al., 2010). Other studies also indicated that simple overexpression of nicotianamine synthase genes may result in increased nicotianamine but not necessarily in augmented Fe utilization by the plants (Cassin et al., 2009). Excessive nicotianamine may restrict the availability of Fe when present in the apoplast (Cassin et al., 2009). It was also found that nicotianamine synthase overexpression can result in increased levels of Fe in leaves, but not consequently in seeds.

In conclusion, it can be stated that increased nicotianamine synthase gene expression can result in beneficial effects on bioavailability of Fe due to the chelator nicotianamine. However, care has to be taken on the site and amount of expression.

4.4 Combination of factors affecting bio-availability of Fe

The above studies suggested that targeting single genes may not necessarily result in an increased level of bio-available Fe. Combining multiple factors that affect bio-availability can be of further advantage. Such approaches have been tested. For example, rice grains expressing *Aspergillus* phytase, bean ferritin and a metallothionein were produced to contain higher levels of Fe in a form that might be bio-available (Lucca et al., 2002). In another study, maize plants were generated that expressed at the same time *Aspergillus* phytase and soybean ferritin in the endosperm of kernels (Drakakaki et al., 2005). These plants had an increased Fe content in seeds by 20-70% and nearly no phytate. Very interestingly, such kernels proved advantageous in bio-availability studies to human cells (Drakakaki et al., 2005).

(Wirth et al., 2009) produced rice plants simultaneously expressing three transgenes, namely a bean ferritin gene, Arabidopsis nicotianamine synthase gene *AtNAS1* and a phytase. Combined ferritin and nicotianamine over-production resulted in a stronger increase of Fe content in the endosperm of grains than was achieved in transgenic approaches with single genes (Wirth et al., 2009).

Thus, attempts to increase bioavailable Fe in seeds are becoming more successful, and combining multiple targets for breeding of Fe efficiency and Fe bio-availability seems to be the key.

4.5 Breeding for novel traits

The above presented approaches rely on the targeting of known components of plant Fe homeostasis mainly in gene technological approaches. An alternative non-transgenic approach is to use the genetic pool of germplasm collections to screen for plant lines that are Fe-efficient and have a high bio-availability of Fe. Such genetic traits can be mapped and backcrossed into the local elite varieties. An advantage of this genetic screening method is that no assumption about the physiology of the traits needs to be made beforehand. Due to the power of modern DNA sequencing the new genes and alleles of interest can eventually be molecularly identified, such as in the case of a transcription factor gene affecting seed micronutrient content (Uauy et al., 2006). In these cases, the power of natural genetic variation is utilized which is based on the natural selection of the best available traits that evolved in the germplasm collection, frequently based on the interplay of multiple genes and specific alleles (quantitative traits). As an example, plant breeders have begun screening for mineral content variation in collections of for example wild wheat (Chatzav *et al*., 2010), rice (Gregorio et al., 2000) and bean (Blair et al., 2010). Furthermore, recombinant inbred lines generated from the original cross of two distantly related inbred lines may help in identifying and mapping of single and quantitative trait loci, for example in wheat (Peleg et al., 2009) and Medicago (Sankaran et al., 2009). In a different approach, cellular Fe uptake and bio-availability analyses have been used to screen rice or maize lines with novel traits not previously associated with known components of Fe usage (Glahn et al., 2002; Lung'aho et al., 2011).

5. Conclusion

Bio-fortification of crops with micronutrients contributes to the improvement of food quality and may help reducing the prevalent disease of Fe deficiency anemia world-wide. Multiple approaches using cereals and other crops have been tested and proven successful. It will

remain as a challenge in the future to further improve details of these procedures, e.g. by exchanging isoforms of the genes, alleles, and new promoters in the case of transgenic approaches. Genetic breeding approaches can be improved by selecting novel recombinant inbred lines and new germplasm for testing. In some studies, the newly generated plant lines have not only been analyzed at plant physiological level for increased Fe content and gene/transgene activity but also for their capacity to augment Fe bio-availability to human epithelial cells (Drakakaki et al., 2005; Zheng et al., 2010) or to cure Fe deficiency anemia (Lee et al., 2009). Such bio-availability studies need to be performed routinely and also used in screening procedures to provide criteria for selection of the best plant lines.

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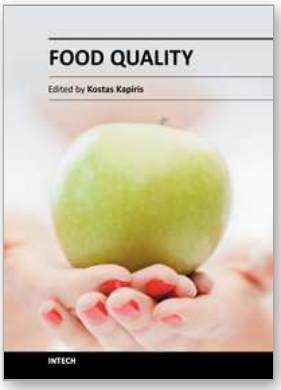

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The book discusses the novel scientific approaches for the improvement of the food quality and offers food scientists valuable assistance for the future. The detailed methodologies and their practical applications could serve as a fundamental reference work for the industry and a requisite guide for the research worker, food scientist and food analyst. It will serve as a valuable tool for the analysts improving their knowledge with new scientific data for quality evaluation. Two case study chapters provide data on the improvement of food quality in marine and land organisms in the natural environment.

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