Micronutrient malnutrition is widespread, especially in poor populations across the globe where daily caloric intake is confined mainly to staple cereals. Rice, which is a staple food for over half of the world’s population, is low in bioavailable micronutrients required for the daily diet. Improvements of the plant-based diets are therefore critical and of high economic value in order to achieve a healthy nutrition of a large segment of the human population. Rice grain biofortification has emerged as a strategic priority for alleviation of micronutrient malnutrition. Nutritional enhancement of crops through conventional breeding is often limited by the low genetic variability for required dietary micronutrient levels. In this case, biotechnology strategies that have been successful in the nutritional enhancement of rice endosperm. These advancements will make substantial contributions to crop improvement and human nutrition. Their practical application, however, also demands visionary changes in regulatory policies and a broader consumer acceptance.

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developing countries (WHO, 2011). Main consequences of iron deficiency include mental retardation, decreased immune function and increased mortality of mother and child at birth (Puig et al., 2007). The severity of the problem for pregnant women is illustrated by the fact that around 200,000 deaths associated with childbirth each year can be attributed to this deficiency (Seymour, 1996). The daily recommended iron intake for human ranges between 8 and 18 mg/day depending upon age and gender, with recommended 30 mg/day for pregnant women (IOM Report, 2011). However, a large number of people in the world do not have the privilege of enriching their diets to allow this recommended intake.

Vitamin-A deficiency is equally important as it can lead to night blindness, xerophthalmia, and to total blindness if remains untreated. Even prior to blindness, vitamin A-deficient children are at a 23% higher risk of death as a result of measles, diarrhea, or malaria (UNICEF, 2009). Adequate supply of vitamin A is important during normal growth and repair of different body tissues and particularly has a vital role in visual system functioning. According to WHO (2009) and UNICEF (2009), vitamin A deficiency is a clinical problem in 45 countries and a sub-clinical problem in 122 countries, with around 190 million pre-school age children and 19 million pregnant women on the globe suffering from it. In some countries of sub-Saharan Africa and South East Asia the incidence is even higher, affecting around 40% of pre-school age children (UNICEF, 2010). Iodine deficiency is often associated with low production of thyroid hormone (hypothyroidism), which plays essential role in normal functioning of the human body. This deficiency can lead to brain damage, and if severe during pregnancy, it can cause stillbirth, spontaneous abortion and other congenital abnormalities (WHO, 2011). Likewise, other micronutrients play vital roles in normal body functioning, and lack of these components in the human diet has severe consequences.

2. Strategies to tackle micronutrient malnutrition

Micronutrient malnutrition can be avoided by dietary diversification, mineral supplementation and food fortification. However, such strategies have not always been successful, mostly for economic or social reasons and/or because of technical difficulties related to the choice of compounds (Frossard et al., 2000; Mayer et al., 2008). For instance, iodine or vitamin C can easily be added to dietary salt whereas addition of folic acid is difficult because of its high water solubility. Iron is one of the most difficult minerals for food fortification, because iron compounds such as FeSO₄ that are most soluble and have high bioavailability are unpalatable and often provoke unacceptable color and taste. Less soluble compounds such as elemental iron, are poorly absorbed by the human body (Hurrell and Egli, 2010). Recently, Hilty et al. (2010) used scalable flame aerosol technology to produce nanosized Fe and Fe/Zn compounds. Based on a rat feeding experiment, these compounds were as bioavailable as FeSO₄ but caused less color change in reactive food matrices than conventional iron compounds used for biofortification. In case of vitamin A, medical supplementation could notably reduce ocular forms of vitamin A-deficiency in many countries (Mayer et al., 2008). Although these vitamin A capsules (which cost around one USD/capsule) have the potential to significantly reduce vitamin A deficiencies, the implementation of this strategy relies a lot on the economic situation of countries (Mayer et al., 2008).

Meanwhile, research has also focused on molecules that promote the bioavailability of micronutrients (e.g., ascorbate and cysteine) and on reducing the activity of antinutrient compounds such as phytate and polyphenolics (Hurrell and Egli, 2010). Other food ingredients such as non-digestible carbohydrates, often termed prebiotics, were found to promote mineral absorption in animal models and in humans (Bouis and Welch, 2010; Hurrell and Egli, 2010; Yeung et al., 2005). Fructans (i.e. the fructo-oligosaccharides), including inulin, have received increasing attention recently (Roberfroid, 2005). They improve systemic absorption and utilization of micronutrients through promotion of beneficial microbiota in the hind gut (Abrams et al., 2005; Dethlefsen et al., 2007), with the potential added benefit that bacteria metabolize antinutrients such as phytate and polyphenols.

The majority of people suffering from micronutrient malnutrition often do not have access to supplementation strategies or possibilities of diversifying their diets. In the long term, therefore, biofortification strategies must include delivery of sufficient micronutrients through locally grown crops that have been optimized for their micronutrient content. Biofortification is feasible for different food crops and complements public health interventions, in addition to being an economical and sustainable solution. Research in crop biofortification is particularly focused on populations that otherwise cannot afford a nutritionally balanced diet in the foreseeable future. Since its official launch in 2004, the HarvestPlus Challenge program is supporting biofortification of staple food crops, including rice, maize, wheat, cassava, pearl millet, beans and sweet potato, primarily for iron, zinc and vitamin A (http://www.harvestplus.org/).

3. Significance of nutritional improvement of rice endosperm

Together with wheat and maize, rice is one of the most important food crops for humans and the main staple food for half of the world population. It provides around 21% of per capita energy and 15% of protein to global human populations (IRRI, http://irri.org/about-rice/rice-facts/rice-basics). Around 3 billion people, mostly in Asia, depend on rice for 35–59% of their caloric intake (Meng et al., 2005) and in many developing countries the dietary contribution of rice is substantially higher. For example, in Cambodia, Myanmar, Bangladesh and Vietnam, rice provides over 70% of dietary energy (Kennedy et al., 2003). This contribution of rice is a success of breeders who over thousands of years made rice productive, high yielding, and an affordable caloric source. However, progress towards improving the micronutrient content of rice has been limited, partially because it was not the focus of rice breeding until recently.

Starch accounts for approximately 90% of total dry matter in rice seeds, followed by a fraction of storage proteins. Furthermore, rice grains are polished since the oil-rich aleurone layer turns the seed rancid upon storage and therefore makes rice unsuitable for consumption. The essential micronutrients which are almost exclusively stored in the husk, aleurone and embryo of rice, are removed during this process. Consequently, polished rice grains contain only small amount of key micronutrients or lack them entirely (Kennedy et al., 2003; Lucca et al., 2006). Importantly, many rice consumers are among the world’s poorest whose diet is largely restricted to rice because it is filling and the most accessible and affordable food. Considering the above facts and the severity of widespread micronutrient malnutrition, improving rice grain (specifically endosperm) nutritional quality would have a significant impact on global human health. This calls for new, high-yielding and high-quality rice varieties containing higher levels of bioavailable vitamins, minerals and essential amino acids for nutrition.

4. Biofortification through plant breeding

Fortification by agronomic practices, e.g. soil fertilization with trace elements, is very difficult for developing countries and technically not feasible in case of complex molecules such as vitamins. Fertilization strategies targeted at improvement of mineral content in cereals grains were partially successful, e.g. in the case of zinc in wheat (Cakmak, 2008; Cakmak et al., 2010) while iron appears to be difficult in this regard. Breeding crop plants for increased levels of promoter substances, such as ascorbate and cysteine to enhance bioavailability of micronutrients, or by reducing antinutrient content (e.g., phytic acid) in their seeds has also been a focus during the last ten years. Mutants with low seed phytic acid content are now available for different staple crops, including rice, wheat, barley and...
maize (Guttieri et al., 2004; Larson et al., 1998; Raboy, 2002). Some of these mutant lines, however, have growth disadvantages. For example, the maize mutant lpa241 with 90% lower phytic acid content also has 30% reduced germination compared to advanced production varieties (Pili et al., 2003). Nevertheless, tortillas prepared from the low phytic acid maize mutant lpa-1-1 increased iron absorption by 49% compared to tortillas prepared from conventional maize varieties (Mendoza et al., 1998).

Since the launch of an initiative by CGIAR in 1992 to increase the mineral content in staple crops (Gregorio et al., 2000; Welch and Graham, 2004), there have been considerable efforts to identify genetic variability in rice accessions for increased grain iron content. In a screen of 939 rice genotypes, almost four-fold differences in grain iron content were observed, ranging between 7.5 and 24.4 µg/g in unpolished grain (Graham et al., 1999). Although results from such studies suggest that a significant potential exists in the rice germplasm to improve micronutrient concentration in the seeds, iron biofortification based on conventional breeding has met with rather limited success. This is in contrast to pearl millet and beans, for which breeding has produced varieties with increased iron content (e.g., lines with more than 100 µg/g iron; Hambidge, 2010). Polished grains of currently cultivated rice mega-varieties have very low iron content (around 2 µg/g; IRRI, 2006). Many years of breeding efforts have achieved only a 2–3 fold increase in iron content, which is insufficient to reach the required target levels of 14 µg/g recommended by HarvestPlus. Even large varietal differences for micronutrients may therefore not be sufficiently significant to improve micronutrient composition of rice grains, particularly considering additional factors such as processing (grain polishing in rice) and bioavailability. Similar to iron, germplasm-screening efforts did not succeed in identifying rice cultivars that accumulate high vitamin A in the grain and variability for other micronutrients is similarly low in the rice germplasm (Beyer, 2010). Together, conventional breeding alone is not an option for micronutrient biofortification in many circumstances and micronutrient improvement of rice endosperm today remains a considerable challenge. Gene technology offers perspectives for efficiently improving the nutritional quality of rice grain to levels of dietary significance.

5. Biotechnology perspectives

Micronutrient biofortification using biotechnology approaches is technically achievable without compromising the agronomic performance of crop plants. Biotechnology allows multi-dimensional approaches, for example, by activation of the metabolic pathways where required and by multi-trait stacking to facilitate introgression into mega-varieties. Various strategies have been reported to improve the nutritional value of the edible parts of different crop plants. Following the development of Golden Rice (Paine et al., 2005; Ye et al., 2000), transgenic approaches to increase provitamin A content have been extended to maize and banana (Aluru et al., 2008; Tanumihardjo, 2010). Metabolic engineering of folate synthesis has been achieved in tomato and rice (Diaz de la Garza et al., 2007; Storozhenko et al., 2007) and engineering of the vitamin B folate pathway is underway in various crops, including soybean, maize and canola (Hirsch, 2009; Zhu et al., 2007), after the successful proof of concept in Arabidopsis (Shintani and DellaPenna, 1998). Similarly, vitamin B6 levels could be increased in Arabidopsis seeds by seed-specific expression of PXDX1 and PXDX2, which are key enzymes in the vitamin B6 biosynthetic pathway (Chen and Xiong, 2009), allowing this technology to be ready for testing in crop plants. Maize endosperm has recently been engineered for simultaneous modification of three metabolic pathways in order to increase beta-carotene, ascorbate and folate (Naqvi et al., 2009), opening the way for the development of nutritionally complete cereals. For this review, we will focus the discussion on the advancements that have been made towards nutritional enhancement of rice grain in particular (Table 1).

5.1. Metabolic engineering for vitamin biofortification in rice

Golden Rice is the best-known example of vitamin-A biofortification in rice and proof-of-concept has also been established for folate (vitamin B9) synthesis in rice endosperm. To achieve these goals, genetic engineering has been a successful strategy because of very low genetic variability available for these traits in the rice germplasm (Beyer, 2010).

5.1.1. Provitamin A biofortification of rice endosperm

Development of Golden Rice by Ingo Potrykus and colleagues was an important breakthrough, demonstrating that the (β-carotene biosynthetic pathway could be engineered in rice endosperm (Ye et al., 2000). Rice plants do not synthesize β-carotene in the grain because key pathway enzymes are only expressed in vegetative tissues. While the isoprenoid precursor geranylgeranyl diphosphate is synthesized in the endosperm, conversion of geranylgeranyl diphosphate to β-carotene in plants requires phytoene synthase (PSY), phytoene desaturase, ζ-carotene desaturase and lycopene β-cyclase. However, targeted expression of a daffodil PSY gene together with the gene for a multifunctional bacterial carotene desaturase (crtI) was sufficient to reconstitute the β-carotene biosynthetic pathway in the rice endosperm (Burkhart et al., 1997; Ye et al., 2000). Since the initial proof-of-concept studies, Golden Rice has been further improved. Paine et al. (2005) demonstrated that PSY from maize together with Erwinia uredovora crtI was the most efficient enzyme combination to increase carotene accumulation up to 37 µg/g in rice endosperm, of which 84% (31 µg/g) was β-carotene. This significant achievement in nutritional biofortification of rice endosperm was a major milestone in combating vitamin A deficiency and its associated effects. Golden rice has now been demonstrated as an effective source of vitamin A (Tang et al., 2009) and studies that focused on Golden Rice in view of public health in India suggest that, with governmental and public support, it has the potential to significantly reduce the disease burden of vitamin A deficiency (Stein et al., 2008).

Several years after its production, Golden Rice has overcome many hurdles and has now been incorporated into breeding programs in India, Indonesia, Philippines and Vietnam, although to date no country has approved Golden Rice for use by farmers. The Golden Rice varieties are bred into local adapted rice varieties that are widely consumed in Asia (Potrykus, 2008) and development of varieties to be released to farmers is underway at public research institutions (www.goldenrice.org).

5.1.2. Engineering higher folate levels in rice endosperm

Folate (vitamin B9) biosynthesis in plants requires the plastid chorismate pathway intermediate para-aminobenzoic acid (PABA) and pterin precursors from GTP in the cytosol. PABA and pterine precursors are then used for folate condensation in mitochondria. Storozhenko et al. (2007) engineered rice using targeted expression of Arabidopsis GTP-cyclohydrolase (GTPCHI) and aminodeoxychorismate synthase (ADCS) to increase folate biosynthesis in seeds. The strategy worked best when GTPCHI and ADCS were expressed together from a single locus, resulting in 15–to 100-fold increases in folate levels in different independent transgenic lines compared to nontransgenic siblings. Plants transformed with ADCS alone had 49-fold increased levels of PABA, but the total folate content in these lines was 6-fold lower than in wild type plants, suggesting that folate biosynthesis in rice may be regulated by a currently unknown feedback mechanism (Storozhenko et al., 2007). Interestingly, this was not the case in tomato fruits overexpressing Arabidopsis ADCS, in which high PABA levels did not reduce folate accumulation (Diaz de la Garza et al., 2007; Storozhenko et al., 2007). Similarly, folate levels
in plants expressing only GTPCI were not significantly different from wild type controls, although pterin accumulation was 25-fold higher in these plants.

Based on cooking experiments, the folate levels in 100 g of rice grains from lines expressing both GTPCI and ADCS in the endosperm are sufficient to meet daily folate needs of an adult individual, even when 45% of folate is lost during cooking (Storozhenko et al., 2007). The bioefficacy, bioavailability, and storage properties of folate-fortified rice were not yet determined in this study. However, the transgenic rice lines have only a low level of polyglutamylated folates (2.6 to 14%) compared to non-transgenic controls (50%), suggesting that folate is bioavailable in the transgenic lines because polyglutamylation negatively affects folate bioavailability. In addition, around 89% of folate in the transgenic lines is present as the 5-methyltetrahydrofolate form, thus making the transgenic biofortification approach superior to industrial folic acid fortification of rice, because consumption of high levels of industrial folic acid may mask the effects of vitamin B12 deficiency (Storozhenko et al., 2007). Further investigations involving field trials will be important to evaluate stability of high folate content and its bioavailability in the field environment.

### 5.2. Biotechnological approaches for iron biofortification of rice

Micronutrient concentrations in plants are tightly controlled by complex homeostatic mechanisms (Palmer and Guerinot, 2009; Puig et al., 2007). Metal ion concentrations in various tissues and compartments are maintained within narrow physiological limits by coordinated uptake, translocation and storage. Since the cellular accumulation of any specific metal ion is a function of uptake capacity and intracellular binding sites, the concentration and affinities of chelators as well as the presence and selective properties of transporters are crucial in metal ion homeostasis (Clemens et al., 2002). Our knowledge on the molecular mechanisms of metal ion transport and homeostasis in plants, and iron in particular, has increased significantly in recent years (see Box 1). Based on the information that is now available for iron uptake and transport mechanisms, several strategies to improve iron content in rice grains have involved Fe uptake and translocation within the plant, in addition to targeting Fe to the endosperm for storage (Fig. 2) (Drakakaki et al., 2000; Lucca et al., 2001; Qu et al., 2005; Takahashi et al., 2001; Vasconcelos et al., 2003; Wirth et al., 2009).

#### 5.2.1. Increasing iron uptake and transport in plants

Rice secretes phytochelatins (Fe$_{3+}$-chelating molecules) in relatively small amounts into the rhizosphere (Walker and Connolly, 2008; Box 1) and is therefore susceptible to low iron availability. Furthermore, the iron concentration in rice leaves is much higher than the amount of iron in polished rice grains, suggesting that re-mobilization of iron from senescing leaves to the seeds might pose a bottleneck in iron translocation to the grain. Rice engineered with nicotiamine aminotransferase (one of the key genes involved in biosynthesis of phytochelatins) produced higher amounts of phytochelatins and was more tolerant to iron deficiency (Takahashi et al., 2001). This demonstrated the capacity of rice to use phytochelatins efficiently, however, the metal ion content in the rice seeds was not discussed in this report.

The control of iron movement in plants might have a substantial role when targeting iron for accumulation within the grain and should be considered for engineering approaches. For example, over-expression of the iron transporter OsIRT1 in rice plants increased iron deficiency tolerance at the seedling stage and also increased iron and zinc concentrations in shoots, roots and mature seeds (although only to a small extent; Lee and An, 2009). However, this approach needs to be optimized. The transgenic plants were sensitive to high levels of Zn and Cd and showed reduced agronomic performance in the paddy field, most likely because the constitutive expression of OsIRT1 under the control of ubiquitin promoter disturbed metal homeostasis. Alternatively, increasing the expression of nicotiamine synthase (NAS) seemed to be a promising approach based on results from an activation-tagged mutant rice line in which OsNAS3 was overexpressed (Lee et al., 2009). The mature seeds of these plants contained increased levels of Fe (2.9-fold), zinc (2.2-fold) and copper (1.7-fold), with similar levels of relative increases in Fe (2.6-fold) and Zn (2.2-
5.2.2. Improving iron bioavailability

In addition to increasing grain iron content, improving the bioavailability of iron is also an important engineering target, i.e., by reducing antinutrient compounds such as phytic acid or increasing promoter substances such as cysteine. Phytate chelates metal ions, which reduces their availability for absorption in the human gut. Degradation of phytic acid in food prior to consumption significantly increases iron absorption (Hurrell and Egli, 2010; Sandberg et al., 1996). For example, Aspergillus niger phytase has been expressed in different transgenic plants including tobacco, soybean and wheat (Brinck-Pedersen et al., 2000); however, this is not a useful approach for most of the cereals because the enzyme is unstable at higher temperatures (>60 °C), and baking or cooking would inactivate the enzyme. For cereals, a thermo-tolerant Aspergillus fumigatus phytase was therefore used to produce transgenic rice (Lucca et al., 2001). The thermo-tolerant enzyme retains 59% of its activity when boiled together with rice, however, the in planta produced phytase showed a strong decrease in its enzymatic activity during cooking (Lucca et al., 2001). For further optimization, different thermo-tolerant phytase sources should be explored to find enzymes that maintain their activity and stability at higher temperatures to sustain cooking. Alternatively, promoter molecules could be expressed in seeds to enhance the bioavailability of iron. For example, overexpression of cysteine-rich metallothionein proteins in rice increased cysteine content of the soluble seed protein amount in comparison to the wild type when tested using a Caco-2 cell model and ferritin synthesis as a cellular biomarker. The performance of the transgenic seeds was similar to the effect of synthetic NA addition to grains prior to in vitro digestion, which also increased Fe bioavailability (Zheng et al., 2010). Although Fe concentrations were slightly higher in the unpolished grain, there were no differences in the Fe content of polished grains between transgenic lines and their non-transgenic siblings (Zheng et al., 2010).

5.2.3. Increased iron storage in the rice endosperm

Ferritin can bind up to 4000 Fe atoms per protein molecule in both plants and humans. Ferritin genes are induced by Fe excess in different plant species such as maize and Arabidopsis, and ferritin iron is soluble and bioavailable similar to iron in ferrous sulfate form (Davila-Hicks et al., 2004). Therefore, strategies overexpressing ferritin in crops have been explored during the past decade. The endosperm-specific expression of soybean or French bean ferritin in rice resulted in two-to-three-fold increases in the grain iron content (Goto et al., 1999; Lucca et al., 2001; Vasconcelos et al., 2003), while its constitutive expression showed iron increases only in vegetative tissues (Drakakaki et al., 2000). These early studies implied that sequestration capacity might be the bottleneck in enhancing endosperm iron content, which could be overcome by higher expression of ferritin. However, subsequent experiments showed that higher levels of ferritin alone do not necessarily increase seed iron content in rice. Even lines expressing the soybean ferritin gene both under the control of the glutenin (GluB-1) and globulin (Glb-1) rice seed storage gene promoters had only a three-fold increase in seed iron levels, as compared to non-transformed controls, although these hyper-expressing ferritin lines had 13-fold increased expression of transgenic protein in the rice endosperm (Qu et al., 2005). Thus, also Fe uptake and transport limit the accumulation of iron in the grains, since the leaf iron concentrations declined to less than half in hyper-expressing ferritin lines in comparison to non-transformed plants (Qu et al., 2005).

5.2.4. Combinatorial approach: evidence of synergistic effect of uptake and storage genes

The studies discussed above imply that expression of exogenous ferritin or single genes responsible for iron uptake and/or transport does not increase the Fe concentration in rice endosperm to required
dietary levels. However, a recently reported strategy combining the genes facilitating Fe uptake and storage in rice showed more promising results. The rice plants constitutively expressing *A. thaliana* Nicotianamine synthase gene (AtNAS) together with endosperm-specific expression of *Phaseolus vulgaris* Ferritin and *Aspergillus fumigatus* Phytase, showed a more than 6-fold iron increase in the polished rice grains (Wirth et al., 2009). The expression of AtNAS1 increased the nicotianamine (NA) content in these plants, which ultimately facilitated iron uptake and transport as Fe³⁺-phytosiderophore chelates. Thus, the synergistic effect of NAS and ferritin was necessary to increase iron content in the rice endosperm (Fig. 1). These transgenic plants had normal agronomic traits under greenhouse conditions (e.g., flowering, yield) and performed better under low Fe conditions, indicating that expression of AtNAS1 and Ferritin did not interfere with Fe homeostasis in these plants. However, these improved rice lines need to be evaluated for performance under field conditions and on iron deficient soils, in order to further confirm the nutritional efficacy of this high-iron rice.

Even with this significant improvement of iron content in rice grains, the recommended levels of daily dietary iron intake by humans (see 1.1, Table 1) required another two-fold increase in grain iron, thus, efforts to further enhance the iron levels need to be continued. Different tissue specific promoter(s) and metal ion transporter(s) combinations should be tested for their efficiency in further increasing rice endosperm iron content.

5.3. Engineering rice grains for increased levels of essential amino acids

Transgenic approaches have also been tested to improve the essential amino acid content in the cereal crops, including rice. Among the exploratory projects, a sulfur-rich seed storage protein from sunflower was over-expressed in rice to increase cysteine and methionine levels (Hagan et al., 2003). This approach reduced the levels of endogenous storage proteins, however, perhaps due to re-allocation of sulfur from the endogenous proteins to the newly added sulfur sink from sunflower (Hagan et al., 2003). In contrast, seed-specific expression of the sulfur-rich seed storage protein from sesame in rice increased crude protein content as well as elevated the levels of methionine and cysteine in the rice grains (Lee et al., 2003). In another approach, the expression of a chimeric tRNA<sub>lys</sub> that incorporates lysine at alternative codons significantly increased the lysine content of rice seed storage proteins (Wu et al., 2003).

Since the synthesis of essential amino acids (such as lysine, threonine) in plants is regulated by complex feedback controls, expression of feedback-insensitive enzymes is another interesting strategy. For example, expression of bacterial aspartate kinase (AK) or dihydrodipicolinate synthase (DHPS) significantly increased the lysine content in the seeds of canola, soybean and maize (Falco et al., 1995; Mazur et al., 1999; Zhu et al., 2007). Alternatively, constitutive expression of a maize feedback-insensitive DHPS raised the free lysine levels by 2.5 fold in the mature rice seeds (Lee et al., 2001), but this effect was not observed when expression of this enzyme was restricted to seeds. Similarly, expression of a feedback-insensitive α-subunit of rice anthranilate synthase (OASA1D) lead to total tryptophan increases ranging between 1.9 and 11.6 fold in seeds (Wakasa et al., 2006). Although the transgenic plants maintained the tryptophan levels under field conditions, their agronomic performance was compromised, including reduced yield, spikelet fertility and seed germination. Since the OASA1D gene was expressed under the control of the constitutive maize ubiquitin promoter, it remains unclear if the seed-specific expression of OASA1D would improve agronomic performance (Ufaz and Galili, 2008). Increases in amino acid and protein content have also been achieved in rice seeds by expressing either rice or *E. coli* aspartate aminotransferase, which is a key enzyme in amino acid synthesis (Zhou et al., 2009). Together, it is possible to increase amino acid levels in the rice grain, but further efforts are required to achieve the increases necessary to meet dietary relevant levels. The additive or synergistic effect of different gene combinations, perhaps, could be the next possibility to explore.
6. Conclusions and future prospects

Considering the dietary needs of humans and farm animals, the nutritional improvement of grains has to become integral part of crop variety development rather than being considered an "added advantage". Thus, interdisciplinary knowledge exchange between nutritionists, plant breeders and biotechnologists is essential. Whether through conventional breeding or gene technology, nutritional improvement of rice seeds requires an integrated understanding of the genetic networks as well as the biochemical and molecular processes that control rice grain filling. It is therefore important to complement rice breeding with functional genomics technologies, including transcript, protein and metabolite profiling, as well as with biotechnological approaches for trait improvement. It has become evident from the examples discussed above, that restricted spatial and temporal expression as well as the appropriate combination of target genes is critical for successful improvement of micronutrient composition in rice grains.

Priority must also be given to the development of rice varieties that combine higher levels of different micronutrients, metabolites and essential amino acids without altering their agronomic performance and eating qualities that make them culturally and socially acceptable. Fig. 3 represents an integrated view of such nutritionally enhanced rice, and considering the achievements to date and strategies underway, this seems possible. Stacking of multiple traits will require efficient gene transfer systems that ensure stable transgene expression over generations without disturbing endogenous metabolic pathways. Single locus insertion of multiple traits would further enhance rice, and considering the achievements to date and strategies ahead: interdisciplinary knowledge exchange between nutritionists, plant breeders and biotechnologists is essential. Whether through conventional breeding or gene technology, nutritional improvement of rice seeds requires an integrated understanding of the genetic networks as well as the biochemical and molecular processes that control rice grain filling. It is therefore important to complement rice breeding with functional genomics technologies, including transcript, protein and metabolite profiling, as well as with biotechnological approaches for trait improvement. It has become evident from the examples discussed above, that restricted spatial and temporal expression as well as the appropriate combination of target genes is critical for successful improvement of micronutrient composition in rice grains.

Current scientific advances must be complemented by public awareness campaigns, however, to promote a better understanding of the biofortification impact and to encourage consumer participation in research for product acceptance. Furthermore, biofortified food production also calls for defining appropriate strategies for product delivery to the most important target populations. If successful, rice biofortification will make substantial contributions to human nutrition by improving health and wellbeing of impoverished populations; however, their practical application also demands changes in current regulatory policies from national and international Food and Drug Administrations to promote broad consumer acceptance.

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