

Biofortifying foods: Tripping over high hurdles

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Abstract. A lofty goal for many in agriculture is the attainment of global nutritional security. Balanced nutrition would help ensure every child an opportunity to thrive. Currently, cereal grains are used as the staple caloric source in most developing countries. Unfortunately, these grains are poor sources of essential minerals (such as iron, zinc and calcium) and vitamin A. Various methods are now being employed to augment these nutrient levels. Proper seed selection coupled with effective soil preparation can increase nutrient levels, while biotechnology approaches have also shown significant promise. While augmenting levels of these nutrients or removing substances that inhibit absorption are important first steps, the true litmus test is whether these higher levels translate into improved absorption and enhanced bioavailability. Utilizing animal models researchers can test the efficacy of foods before conducting the expensive and often controversial human feeding trails. In this review we go over the biofortification efforts that have been undertaken to improve iron, zinc, calcium, and vitamin A levels and to decrease antinutrient components. In addition, we discuss the research done to assess how these changes impact absorbability in humans. Lastly, we discuss the scientific and political issues surrounding implementation of these modifications.

Keywords: Biofortification, micronutrients, antinutrients, genetically modified organism.

INTRODUCTION

People throughout the world use cereal crops for the bulk of their caloric intake (Chassy, 2010); however, a majority of these cereals are low in bioavailable micronutrients such as iron (Fe), zinc (Zn), and vitamin A (Beyer, 2010; Gomez-Galera et al., 2010; Hotz, 2009; Potrykus, 2010a; Stein et al., 2006). Micronutrients are defined as chemical elements or substances required in trace amounts for the normal growth and development of living organisms. There are several methods to improve the content of these limiting compounds within edible portions of plants. Traditional breeding is a proven approach but has several limitations including the protracted time frames needed to breed varieties and the relatively low "micronutrient ceilings" among varieties in a given region (Bonneuil, 2006; Johnson and Veilleux, 2010; Rommens et al.,

2005; Rommens et al., 2004; Wollenweber et al., 2005). Another technique is to supplement the plants with fertilizers or other chemicals to boost their nutrient content during growth (Dodd et al., 2010). This approach is often expensive and time consuming thus limiting large scale application. In addition, you have the secondary consequences of fertilizer burden and chemical run-off if one does not maintain regimented farming practices. In our opinion, the most promising methods are the use of genetic manipulations to increase the micronutrient content, or remove substances that impede absorption (Botha and Viljoen, 2008; Dayod et al., 2010; Gartland et al., 2013; Gomez-Galera et al., 2010; Herrera-Estrella et al., 2005; Johnson et al., 2011; Tang et al., 2009; Wollenweber et al., 2005). One of the benefits to this type

of approach is the ability to relatively quickly produce new varieties without costly time spent screening generations of plants for the desired phenotype. Another advantage is the ability to maximize the nutrient deposition into the edible portions of the food (Aung et al., 2013; Herrera-Estrella et al., 2005; Johnson et al., 2011; Wei et al., 2012a). While molecular biologist favor these techniques for the ability to dramatically alter crop plants, the disadvantages arise from the social and political barriers currently restricting acceptance of genetically modified plants. These hurdles constitute obstacles to improving nutrition in various crops (Potrykus, 2010a; b; Rommens, 2007; Weale, 2010). In addition, determining that the modifications to the plants impart a true human nutritional benefit is an equally important component in all efforts to improve crops (King, 2002). Alterations in the composition of crop plants and how these changes are utilized by the consumer create a complex nexus that impacts the bioavailability of the increased nutrients within consumed foodstuffs.

SCIENCE OF FORTIFICATION - UTILIZING SELECTIVE BREEDING, SOIL AUGMENTATIONS AND GENETIC MODIFICATIONS

As was mentioned in the introduction, there are several ways to fortify nutrients in plants. Here, we will focus on how those efforts have been utilized to improve the content of mineral and non-mineral nutrients. Traditional selection and hybridized breeding have been around for thousands of years (Rommens, 2007). Yield, color, taste and numerous other aspects have been evaluated and the most desired traits selected and cultivated (Hazel and Lush, 1942; Janick, 2005). Not until more recent times could this practice be employed for nutritional factors. Once robust analytic techniques evolved to measure these parameters, soil augmentation and fertilization could be used to reproducibly increase nutrient content. Within the last 25 years the use of specific genetic modifications to enhance and incorporate highly specific genes to impart enhanced accumulation of desired nutrients has pushed the field forward (Cressey, 2013; Herrera-Estrella et al., 2005).

Minerals

About half of world's population depends on cereal food including rice, wheat, and corn, or various legumes as their staple calorie source (Johnson, 2013; Loftas et al., 1995). Given that iron (Fe) and zinc (Zn) are present at low levels in the processed cereals, especially in rice, they are the most common mineral deficiencies seen throughout the world (Bhullar and Gruissem, 2013). Other mineral deficiencies, such as calcium (Ca), magnesium (Mg) and iodine (I) are also prevalent. In the case of I, increased use of iodized salt in non-industrialized

countries has proved to be a huge success (www.micronutrient.org). For other minerals, a much more involved process of food fortification has been undertaken (Aluru et al., 2008; Aluru et al., 2011; Bashir et al., 2006; Chen et al., 2008; Gomez-Galera et al., 2010; Hawthorne KM, 2009; He et al., 2013; Johnson et al., 2011; Lee et al., 2009; Masuda et al., 2013; Naqvi et al., 2009; Paine et al., 2005; Park et al., 2005a; Park et al., 2005b; Park, 2004; Wei et al., 2012a; Wirth et al., 2009; Ye et al., 2000; Yuan et al., 2013; Zhang et al., 2010).

Iron

A World Health Organization (WHO) report shows that Fe deficiency affects an estimated two billion people, causing almost one million deaths annually worldwide (WHO, 2013). The primary symptom of Fe deficiency is anemia, which leads to extreme fatigue, weakness and increased heartbeat. Iron is also needed for proper fetal, infant and child development (Mayer et al., 2008; WHO, 2013). To cope with this nutrient deficiency, several strategies have been implemented to boost Fe concentrations in the edible part of the crops, or to lower the level of Fe-chelating antinutrients to enhance Fe bioavailability.

The first strategy is through foliar application of Fe-rich fertilizers. Several studies report that Fe content in rice can be increased from 14.5 to 37.1% in polished grain, depending on the types of applied Fe-fertilizers (Fang et al., 2008; He et al., 2013; Wei et al., 2012b; Yuan et al., 2013). Another study with winter wheat reports that whole grain wheat Fe concentration are significantly increased by 22% with an application of FeSO₄-containing fertilizers (Zhang et al., 2010). Even though a large part of Fe is lost by milling (the process of removing the outer bran from the starch filled inner layer of the grain), the flour Fe content still has a significant increase from 10.4 to 12.4 mg kg⁻¹ (Zhang et al., 2010). Overall, these studies show foliar spray can be an effective method to boost Fe content in plant foods. However, in many developing countries this approach may be financial impractical due to the high costs associated with Fe-fertilizers. In addition, there is a limited capacity to increase the Fe content utilizing this methodology mainly because the majority of Fe in the grain is allocated to the seed coat. Nonetheless, in areas where soil Fe is low, biofortification through fertilization may be necessary, and can be used in combination with other biofortification strategies.

The second strategy is through selection of high-Fe crop varieties and then combining the high-Fe phenotype with other desirable characteristics through conventional breeding. HarvestPlus, a non-governmental organization, has carried out multiple breeding efforts to create high-Fe crops including beans and pearl Millets (Kodkany et al., 2013). In these efforts the Fe content in the edible part of the plant is increased up to 2 fold (Cercamondi et al., 2013;

Tako et al., 2011). To date, reports on the breeding of high-Fe rice are rare due to the lack of sufficient genetic variation.

The third strategy relies on modern biotechnology to create genetically modified (GM) crops. Iron is an essential element for plants, playing critical roles in respiration, chlorophyll biosynthesis and photosynthetic electron transport (Marschner, 1995). Iron uptake, homeostasis, transport and storage in plant organs are tightly controlled by various transporters and cellular regulators (Marschner, 1995). By genetically engineering these Fe regulating factors, it is possible to enrich Fe in the edible part of the crops. Wirth and colleagues created a transgenic rice line that harbors two transgenes, nicotianamine synthase (NAS) and ferritin. Nicotianamine synthase is an enzyme involved in the synthesis of Fe³⁺-chelating compounds important for Fe absorption from low Fe soils, thus enhancing Fe uptake from low Fe soils (Bashir et al., 2006; Wirth et al., 2009). Ferritin is the principal Fe storage protein in all aerobic organisms and can store up to 4500 Fe(III) atoms in its cavity in a soluble and bioavailable form (Harrison and Arosio, 1996). Thus the over-expression of ferritin in the rice endosperm can lead to increased bioavailable Fe storage capacity in the rice seed (Hell and Stephan, 2003; Ling et al., 1999; von Wiren et al., 1999). Wirth and colleagues show that the over-expression of NAS and ferritin conferred a synergistic effect in Fe uptake and storage and successfully increased the Fe content of the rice endosperm by more than six-fold. Moreover, the high Fe rice line does not appear to have any significant loss in yield or other phenotypic changes, except for a tendency for early flowering (Wirth et al., 2009). Though successful, the above effort was not able to increase Fe in rice to 14.5 µg/g, the target concentration that nutritionists have recommended to meet Fe requirements in populations consuming a rice-based diet (Hotz et al., 2012; Lucca et al., 2002). However, Johnson and colleagues did surpass the target with their transgenic rice lines harboring a single transgene, one which increases the Fe chelating capacity (Johnson et al., 2011). These plants achieve Fe concentrations up to 19 µg/g in polished grains.

More recently, Masuda and colleagues have created transgenic rice lines harboring a soybean ferritin gene and the barley NAS gene, along with three other genes involved in the synthesis of Fe³⁺-chelating compounds. Their analysis showed that the concentration of Fe in polished seeds was increased by 4 and 2.5 times, in high Fe or low Fe conditions, respectively. A notable feature of this transgenic rice is that it was created without the use of antibiotic selection to alleviate public concerns (Masuda et al., 2013). We will touch on the significance of this modification later in the review. A previous report has documented examples where ectopic ferritin expression in the leaves under Fe deficiency conditions might cause the accumulation and sequestration of Fe, leading to Fe deficiency-related growth defects (Van Wuytswinkel et al., 1999). However, introduction of the

three extra biosynthetic genes for Fe³⁺-chelating compounds relieved these deleterious phenotypes in the plant (Masuda et al., 2013). This is an important factor to consider because modifications of plants to enhance biofortification of nutritional components should not compromise plant health.

Iron-containing plant hemoglobins are another promising target for altering Fe content in plant-based foods. Plant hemoglobin is similar to the human hemoglobin, with Fe binding capacity, and is most commonly found in nodulating legumes (nitrogen fixing plants) (Kundu et al., 2003). Soybean leghemoglobin appears to be as bioavailable as bovine hemoglobin (Proulx and Reddy, 2006), and the absorption of heme Fe is not affected by dietary factors that interfere with the absorption of nutrients (Lynch et al., 1985). Multiple efforts are underway to map the genomic regions associated with seed Fe contents in legumes and rice (Anuradha et al., 2012; Blair et al., 2013). It is foreseeable that these studies could generate more target genes for genetic modifications to improve Fe content in the future.

Zinc

Zinc is an essential micronutrient serving as a cofactor for almost 300 enzymes (Sinclair and Kramer, 2012). Insufficient Zn uptake in humans can cause stunting in children, impaired immune function, and adverse pregnancy outcome in women (Brown et al., 2004; Hess and King, 2009). Zinc bioavailability is limited in major staple foods such as rice, legumes, and cereals mainly due to the presence of antinutrients such as phytate (Lonnerdal, 2000). It is estimated that Zn malnutrition is responsible for about 4% of the worldwide morbidity and mortality of young children (Bryce et al., 2005). Thus improving Zn bioavailability in various crops is of paramount importance.

Zinc supplementation is an effective approach to prevent Zn deficiency in at-risk populations, but the infrastructure required for successful implementation is often lacking in developing countries (Brown et al., 2009). Biofortification through agronomic interventions by foliar application of Zn-rich fertilizers is a promising alternative. In a field study with rice, Wei and colleagues validated the efficacy of ZnSO₄ and Zn-amino acids as additives in fertilizers to increase Zn levels in rice grain. Their report showed that on average, Zn-amino acid and ZnSO₄ increased Zn concentration in polished rice up to 24.04 and 22.47%, respectively; and increased Zn bioavailability in polished rice up to 68.37 and 64.43%, respectively (Wei et al., 2012a). Conventional breeding of several high-Zn crop varieties are underway. It is estimated that in 2013, through HarvestPlus, high-Zn rice and wheat will be released in India, Bangladesh and Pakistan (www.harvestplus.org).

Genetic modification efforts targeted solely for Zn biofortification have been rare. However, since the common

target gene in Fe biofortification, NAS, can regulate uptake of other transition metals including Zn, and Copper (Cu), NAS overexpressing crops usually accumulate higher Zn in the seeds (Aung et al., 2013; Johnson et al., 2011). For example, a rice line that overexpresses NAS and ferritin, shows 1.3 more Zn increase in addition to 3.4-more Fe (Aung et al., 2013). Many other plant genes have now been characterized in Zn uptake, transport, partitioning and homeostasis. Possible candidate genes for genetic modification-based biofortification include, but are not limited to, genes that mediate the accumulation of Zn into vacuoles (Menguer et al., 2013) and Zn transporters that mediate low affinity Zn transport in vascular tissues (Tiong et al., 2013).

Calcium

In humans, Ca is an essential nutrient needed for many biology processes including adequate bone health, and vitamin D absorption (Heaney, 2013; McDaniel and Williams, 2013; Weaver and Heaney, 2006). Within the plant Ca is also important for structural integrity (Dodd et al., 2010; Galon et al., 2010). The two most effective means to increase dietary Ca within plants has been expression of transgenes and mutations within gene(s) controlling the formation of the antinutrient oxalate crystals (Kim et al., 2006; Kim et al., 2005; Morris et al., 2008; Morris et al., 2007; Park et al., 2005a; Park et al., 2005b; Park, 2004). The primary methodology behind this increased Ca was the incorporation of an activated membrane Ca transporter termed sCAX1 (Hirschi, 1999). Overexpression of this constitutively active transporter more than doubled the Ca content in carrots and tomato fruit and as much as three fold more Ca in potato tubers (Morris et al., 2008; Park et al., 2005a; Park et al., 2005b; Park, 2004). This change did cause deleterious effects in tomatoes including stunted growth and reduction in yield; however, this modification does not appear to alter yield in carrots and potatoes. Recently, these high-Ca content tomatoes were modified with a Ca binding protein (from maize) that compartmentalizes Ca in a different part of the cell and alleviated some of the deleterious effects (Wu et al., 2012). Utilizing a slightly different Ca transporter, tomato fruit Ca was increased 50% but there were no deleterious plant characteristics (Park et al., 2005a). This transgene incorporation proves that significant gains in fortification of Ca can be achieved in crops, although diligent care needs to be taken to decrease harmful effects on plant growth.

Non-minerals

Without a doubt the most widely known non-mineral fortification project in world has been that of low vitamin A in rice (Ye et al., 2000). As rice is a staple in the diets of

almost 42% of the world's population, many of them in poor and developing countries, combating the blindness and other developmental deficiencies resulting from low vitamin A content rice has been a concern of the WHO for almost 20 years. Due to the controversy and low acceptance of GM food, there are also multiple successful efforts to boost Vitamin A content in plant food through traditional breeding. To date, varieties with high Vitamin A in sweet potato (Hotz et al., 2012), cassava (Talsma et al., 2013) and corn have been bred (www.harvestplus.org).

Golden Rice was developed by incorporating genes involved in the β -carotene (the precursor of vitamin A) synthesis pathway (phytoene synthase and carotene desaturase) into the rice endosperm (Ye et al., 2000). Subsequently, Golden Rice 2 has been created which has over 20 times more β -carotene than the original modified variety (Paine et al., 2005). Expressing these genes in the rice endosperm creates kernels fortified with β -carotene. The success of this process could lead to development of crops with enrichment of other limiting vitamins, for instance vitamin E.

Vitamin A rich corn has also been generated through overexpressing β -carotene synthesis pathway genes. Aluru and colleagues have generated transgenic corn that contains up to 34-fold total carotenoids in the endosperm compared to wildtype (Aluru et al., 2008). More recently, transgenic corn plants have been created in which the levels of 3 vitamins which increased simultaneously in the endosperm through the modification of 3 separate metabolic pathways (Naqvi et al., 2009). The transgenic kernels contained 169-fold the normal amount of β -carotene, 6-fold the normal amount of ascorbate, and double the normal amount of folate (Naqvi et al., 2009). This is an excellent example to showcase the power and efficiency of modern biotechnology to transform nutrition.

Neutralizing the anti-nutrients

While we have discussed methods to fortify minerals and vitamin A, reducing antinutrient content is another method to improve available nutrients. Plants contain antinutrients, compounds that interfere with the normal absorption of nutrients. Phytic acid (PA), oxalic acid, oxalate crystals and glucosinolates all have deleterious effects on numerous micronutrients (Hambidge et al., 2005; Heaney and Weaver, 1990; Ishida et al., 2003; Weaver et al., 1987). Traditional breeding and varietal selection have succeeded in decreasing the levels of these compounds; however, these compounds have roles in plant defense and development thus complete removal is problematic (Nakata and McConn, 2000; Schroeder et al., 2013). To that end understanding the interactions of these compounds with micronutrients is vital to the ultimate goal of improving the nutritional availability of micronutrient with plants.

Phytic acid

Phytic acid or the salt version phytate is a highly phospho-rich compound found in plants. Mostly found in seeds and grain they serve as a phosphorus pool and energy for the plant. While this is highly beneficial to the plant it creates a deleterious effect for humans. This acid can chelate minerals and decrease their absorbability, notably for Zn, Fe, Ca and Mg (Manary et al., 2002; Raboy, 2002, 2007). Several natural or genetically mutated plant varieties show dramatic decreases in PA. It was shown that the absorption of Ca from tortilla meals prepared from low-phytate maize was significantly increased by over 50% compared with that from meals prepared from maize with typical phytate content (Hambidge et al., 2005). Through the expression of *Aspergillus niger* phytase, an enzyme that catalyzes the hydrolysis of PA into inorganic phosphorus, reduction of phytate in kernels has been achieved in maize, without the undesired agronomic effects seen in low-phytate mutants (Chen et al., 2008). Additionally, current genetic testing for PA seed contents can yield valuable information for future engineering of crops with optimized seeds PA content and enhanced mineral bioavailability (Sompong et al., 2012). Another interesting approach is to remove the PA once the food stuff is consumed. The genetically modified Enviropigs produce the enzyme phytase in their salivary glands. When cereal grains are consumed, the phytase mixes with feed in the pig's mouth, and once swallowed the phytase is active in the acidic environment of the stomach degrading indigestible PA with the release of phosphate that is readily digested by the pig (Forsberg et al., 2013).

Oxalates

Like PA, oxalate or oxalic acid (OX) is found in plants and serves primarily as a defense mechanism to reduce consumption by predators by forming crystals incorporating Ca. These oxalate crystals while beneficial to the plant render the Ca unabsorbed in humans (Heaney and Weaver, 1989). Low oxalate levels in plants generally correlate with high Ca absorption. For instance kale (low OX) (Weaver et al., 1987) has much higher bioavailable Ca level, compared to spinach (high OX) (Weaver et al., 1987). Although OX is not as common in many of the highly consumed cereal grains, its presence in other foods widely consumed in poor countries, such as cassava, makes reduction of OX another avenue to improve fortification of Cain worldwide foodstuffs. A mutation in *Medicago truncatula* is deficient in the formation of oxalate crystals (Nakata and McConn, 2000) without altering the Ca concentration (Morris et al., 2007; Nakata and McConn, 2000). Identifying these crystal forming genes could lead to new strategies to alleviate inhibition of Ca absorption from plants with high oxalate levels.

UNDERSTANDING THE NUTRITIONAL IMPACT – USING VARIOUS MODEL SYSTEMS TO ASSESS BIOFORTIFICATION EFFICACY

All of these modifications have created a battery of plants with increased nutrient content. However, the ultimate question still remains, are these improvements (Table 1) nutritionally beneficial? To assay absorption researchers can utilize several techniques and model organisms. One of the most common methods employed is the use of isotopic tracers. In mineral bioavailability studies, one can use intrinsic labeling with either stable isotopes (non-radioactive) or radioactive isotopes (Hawthorne, 2009; Morris et al., 2008; Morris et al., 2007; Tang et al., 2012; Tang et al., 2009; Weaver and Chaney, 1985; Weaver et al., 1987). When it comes to measuring absorption of compounds from fortified plants, intrinsic labeling is a much more accurate measure of true nutritional bioavailability. To accomplish this, plants need to be grown in a hydroponic system to maximize uptake of expensive labels (Morris et al., 2008; Tang et al., 2012; Tang et al., 2009). The mineral tracer of interest is incorporated into the plant as if it was the natural element but has the unique property that it can be measured separately of the endogenous element (Figure 1). This has tremendous analytical benefits as the tracer is incorporated within the plant tissues, cellular compartments, and conjugated normally throughout the growth cycle of the plant (Weaver and Chaney, 1985). In comparison, utilizing extrinsic labeling offers quicker diet preparation of labeled prepared foodstuffs (Figure 1). Here, the plants are grown in their normal or experimental condition (such as augmented with fertilizer or foliar application), harvested and processed under normal methods. The prepared meal is then mixed with tracer prior to feeding. Utilizing extrinsic labeling is useful when assessing efficacy of some antinutrients, primarily PA and places where inadequate control of plant growth exists (Adams et al., 2002; Hambidge et al., 2004; Hambidge et al., 2005; Jang et al., 2003; Li et al., 2000; Mazariegos et al., 2006; Mazariegos et al., 2010; Mendoza et al., 1998). However, there are limitations, as this technique does not mimic normal tissue and cellular localization and does not accurately portray any chemical conjugation which may occur in plants designed to have higher mineral concentrations, which could lead to inaccurate absorption measurements (Andon et al., 1993; Boza et al., 1995; Weaver et al., 1987). Another assessment technique is to utilize fractional absorption calculations when utilizing intrinsic or extrinsically labeled diets (Figure 2). This involves utilizing two isotopes of the same tracer (which can increase costs considerably) where one is utilized in labeling the food and the other is injected intravenously (Abrams et al., 1994; Moser-Veillon et al., 2001; Patterson and Veillon, 2001; Yergey et al., 1994). By knowing the amounts of tracers and analyzing either urine, fecal or both materials the amount of orally ingested tracer can be compared to that of the

Table 1. Nutrient bioavailability tests of fortified crops.

Crop	Nutrient	Method of modification	Nutrient absorption tested	Reference
Rice	β -Carotene	Genetic modification	Humans	Tang et al. (2012)
Corn	β -Carotene, Vitamin C, Folate	Genetic modification	Not reported yet	Naqvi et al. (2009)
Sweet potato	Vitamin A	Traditional breeding	Humans	Hotz et al. (2012)
Cassava	Vitamin A	Traditional breeding	Humans	La Frano et al. (2013)
Rice	Fe (with Zn) Fe, Zn	Genetic modification	Not reported yet	Masuda et al. (2012) Aung et al. (2013) Johnson et al. (2011)
Bean	Fe	Traditional breeding	Humans, absorption unchanged despite higher Fe content	Blair et al. (2010) (breeding) Petry et al. (2012) (feeding)
Corn	Fe, low phytic acid	Genetic modification, Traditional breeding	Caco cells, humans	Aluru et al. (2011) Lonnerdal et al. (2011)
Wheat	Fe	Traditional breeding	Caco cells	Salunke et al. (2011)
Pearl millet	Fe, Zn	Traditional breeding	Humans	Kodkany et al. (2013) Cercamondi et al. (2013)
Rice	Zn	Traditional breeding	Not reported	HarvestPlus News
Wheat	Zn	Traditional breeding	Not reported	HarvestPlus News
Corn	Vitamin A	Traditional breeding	Not reported	HarvestPlus
Carrots, Potato, Tomato	Ca	Genetic modification	Mice, humans (carrot only)	Morris et al. (2008)

injected sample (which represent 100% absorption – thus any excreted amount will infer each person's individual retention ability for the particular compound). In addition, utilization of a third tracer, comparisons can be made to foodstuffs with known absorbability of your test compound (for instance – Ca from milk: Figure 2). By comparing the known concentration of the tracer in the urine, to that of tracer ingested from the labeled test meal, and/or foodstuff with known absorbability, one can produce a fractional

absorption for the orally ingested tracer mineral (Abrams et al., 1994; Hawthorne KM, 2009; Yergey et al., 1994). Together these techniques can be employed to determine the effectiveness of the biofortification process when coupled with various models (Table 2) on which we elaborate as follows.

Cell lines

Caco-2 cells (derived from a human colon

carcinoma – can mimic intestinal epithelial cells: forms polarized monolayers, defined brush borders and intercellular junctions) are widely used in nutritional studies (Sambuy et al., 2005). The Caco-2 cell model coupled with *in vitro* digestion is an economical and efficient way to quickly survey bioavailability of nutrients from a large number of varieties. In the case of Fe, a measure of ferritin formation serves as a marker for Fe uptake (Aluru et al., 2011; Tako et al., 2013) and for Zn, a digested and radio labeled

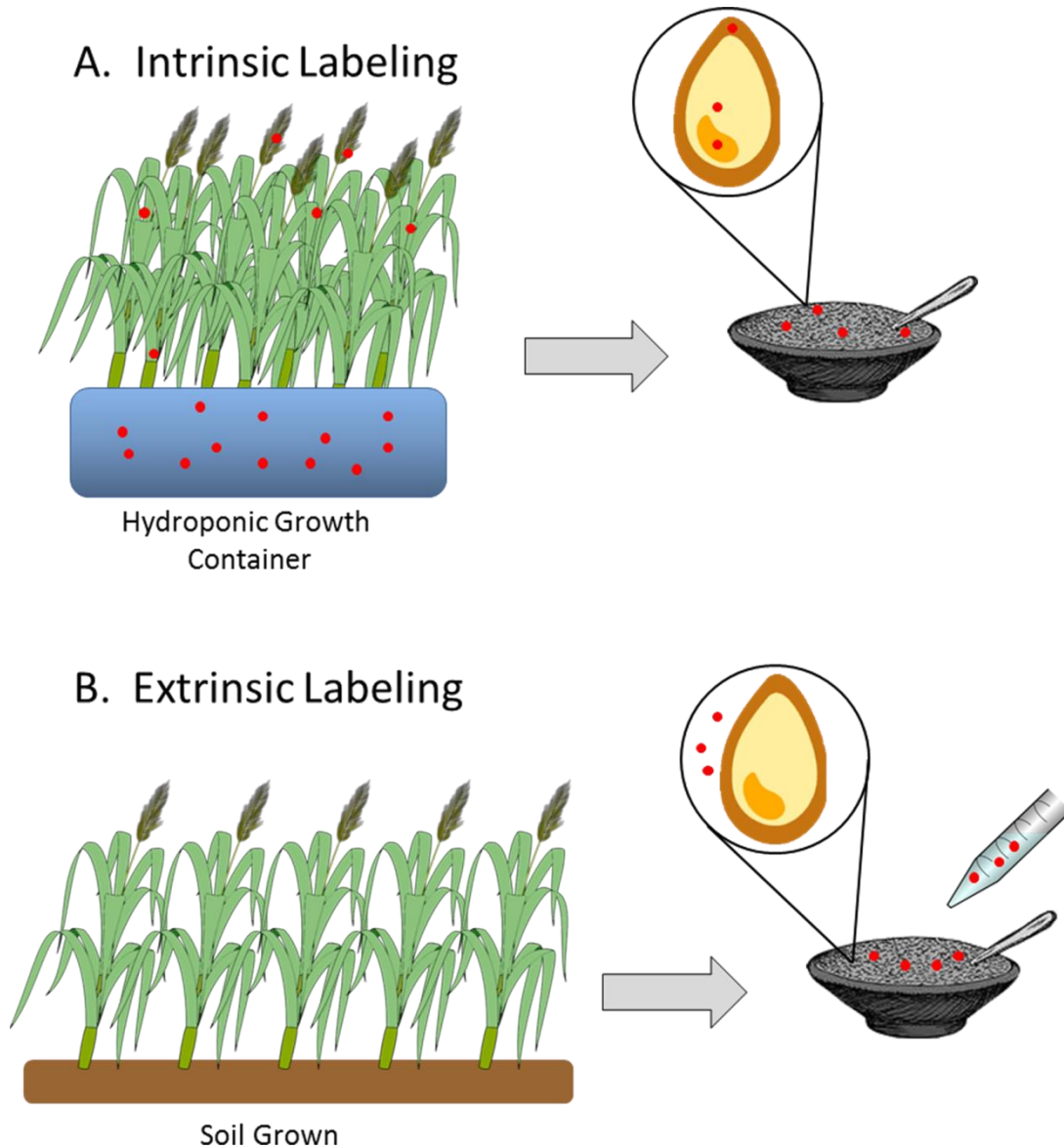


Figure 1. Isotopic Labeling Regimes.

A. Intrinsic labeling of plants – isotope tracer (red circle) is added to hydroponic growing solution, absorbed through the roots and deposited throughout the plant, including the edible grain.

B. Extrinsic labeling of plants – plants are grown, harvested and processed in their normal or experimental regime, foodstuff (grain) is harvested and prepared for testing, label is mixed with foodstuff prior to ingestion by test subjects.

rice solution was used to treat cells and total Zn uptake measured (Jou et al., 2012). Bodnar and colleagues created a transgenic maize line harboring a hemoglobin (utilizing a fluorescent protein tag to track the hemoglobin movement) and showed in Caco-2 cell-culture conditions that Fe in ectopically expressed hemoglobin was as bioavailable as ferrous sulphate, suggesting the possibility of using hemoglobin as an effective biofortification regime for Fe in crops (Bodnar et al., 2013). It might be possible for other biofortified constituents to be measured in a similar fashion using

this cell line but processing, labeling and cell viability testing will need to be determined for the specific plant material and constituent combination. However, the lack of microflora and other large intestine factors limit the accuracy of this system compared to *in vivo* models.

Animals

Animals have been utilized to determine the efficacy of biofortified plants by numerous researchers (Li et al., 2012;

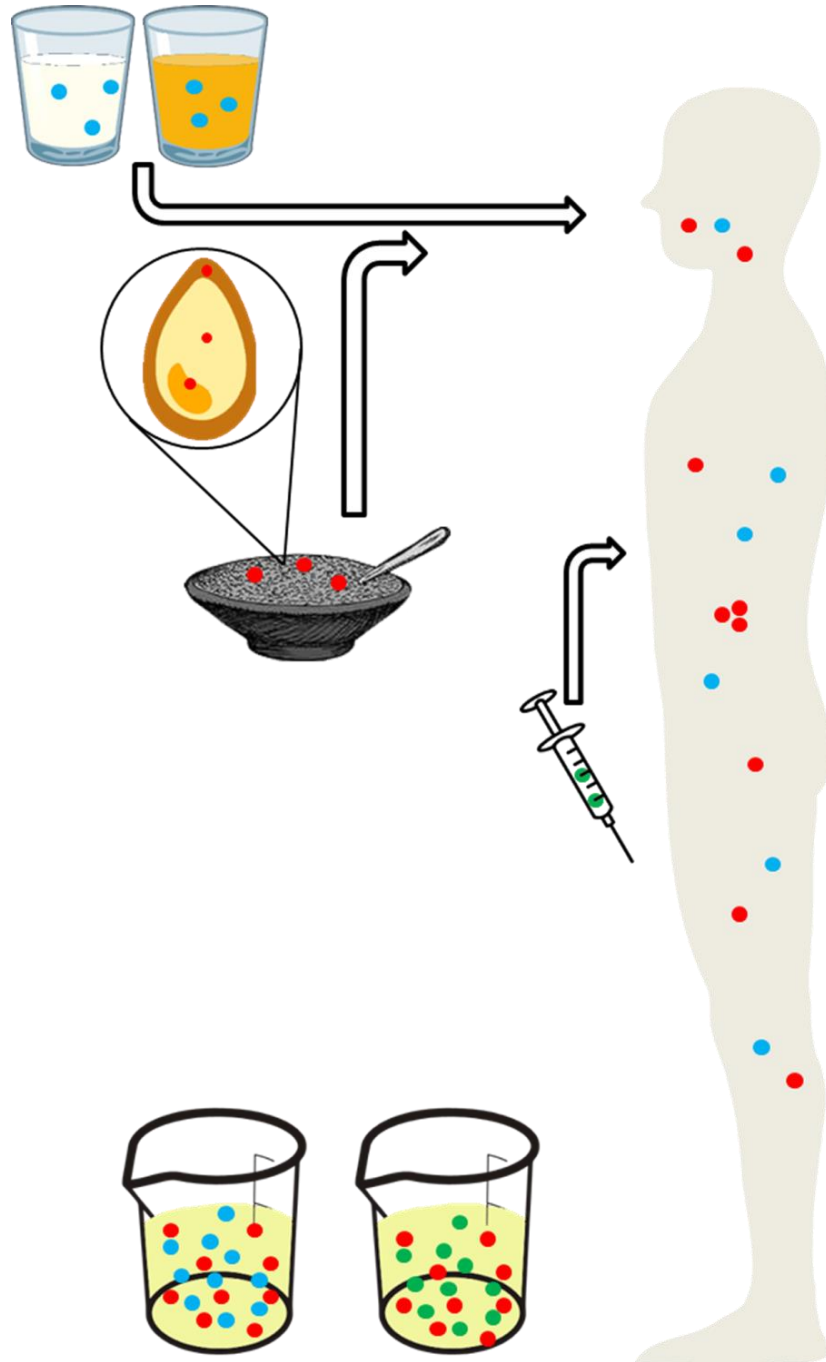


Figure 2. Dual Tracer Fractional Absorption.

In this diagram, the example will be intrinsically labeled grain with calcium as the mineral under study. Stable isotope labeled meals are consumed in a normal manner (red circle – ^{42}Ca) 2. Subjects consume a second stable label (blue circle – ^{48}Ca) in milk or orange; and/or subjects can be given isotope by IV (green circle – ^{46}Ca) 3. Urine and/or feces is collected and the ratio of two tracers is used to infer absorption of calcium from the meal or to compare absorption of calcium to that of absorption from food with a known percent absorption, calcium from milk.

Lonnerdal et al., 2011; Mills et al., 2008; Morris et al., 2008; Morris et al., 2007). Rodents are the most popular animal model followed by pigs and chickens. Obviously,

these animals all afford an economical alternative to human trials. In the case of genetically modified (GM) foods, as we will detail in a later section, it also reduces

Table 2. Models of used to evaluate biofortification efficacy.

Model	Nutrient(s) or antinutrient	Crop	Reference
Caco-2 cells	Fe, Zn	Rice, corn, bean	Ariza-Nieto et al. (2007), Jou et al. (2012)
Domestic chicken	Fe, phytate	Rice, corn, bean,	Jang et al., (2003), Tako et al. (2011); Tako et al. (2013)
Mongollan gerbils	Vitamin A	Carrots	Mills et al. (2008)
Mice	Ca, oxalate	Carrots, medicago	Li et al. (2012), Morris et al. (2008), Morris et al. (2007)
Rat	Zinc	Rice	Jou et al. (2012)
Piglets	Fe	Bean	Tako et al. (2009)
Humans	Fe, Zn, Ca, Vitamin A, phytate	Rice, carrots, corn	Hambidge et al. (2004), Hambidge et al. (2005), Lonnerdal et al. (2011), Mazariegos et al. (2006), Mazariegos et al. (2010), Morris et al. (2008), Petry et al. (2012), Tang et al. (2012), Tang et al. (2009)

the controversy. Animals can be fed diets depleted in minerals or vitamins and/or fasted prior to ingestion of the fortified foodstuffs (Mills et al., 2008; Morris et al., 2008; Morris et al., 2007) to insure complete consumption of labeled diets. In the case of Mongolian gerbils, their diet was depleted in carotenoids and vitamin A to deplete liver retinal stores (Mills et al., 2008). These types of strict diet modifications are difficult to impose on human subjects. After the 4 week diet depletion, feeding with different varieties of carrots with increased vitamin A levels show improved liver retinal stores and serum antioxidant compared to control diet fed gerbils (Mills et al., 2008). In the case of animals, cheaper radioisotopes can be used in labeling regimes along with direct measurements of nutrient incorporation into tissues. More recently, Lonnerdal and colleagues evaluated the bioavailability of Zn in seeds of low phytic acid (lpa) variants of corn, rice, and barley using a suckling rat pup model. This model is very sensitive to PA content in the diet (Lonnerdal et al., 1989; Sandstrom et al., 1983). Utilizing extrinsically radio labeled Zn in the diets they found that the reduction of PA causes an increase in Zn absorption from both corn and rice (Lonnerdal et al., 2011). This is a substantial increase compared to Zn and Fe studies from lpa corn in human studies and suggests that results in rats might not always correlate to those studies in humans. In mice fed carrots intrinsically labeled with a radioisotope of Ca, the deposition of Ca into the hind legs is similar between control plants diets compared to diets containing half as much of the modified carrot (Morris et al., 2008). The same was true when using an extrinsic labeling regime in these mice. However, similar results between the two labeling regimes are not always observed. In oxalate deficient Medicago plants only the intrinsically labeled plants showed an increase in Ca bone deposition compared to diets containing extrinsically labeled Ca (Morris et al., 2007) suggesting that extrinsic labeling might not accurately assess the potential of fortification efforts designed to reduce antinutrient levels. Assessing Ca deposition in the bone of mice adds to the research by investigating the biological function of Ca from the modified plants and not just changes in absorption.

Combined with the utilization of animals which mimic different diseases or impaired absorption of minerals which could serve as proxies for malnourished populations, these features can improve the understanding of biofortification regimes. For instance a recent study showed that reduction of Ca oxalate in plants, along with increased Ca bone deposition, could rescue the phenotype of mice deficient in the vitamin D receptor (this disrupts Ca absorption and decreases bone density) (Li et al., 2012). In a study to assay bioavailability of Fe in GM rice, rats were fed a Fe depleted diet for 2 weeks. Then three transgenic rice varieties, with increased Fe accumulation, were fed to rats and showed no differences in Fe bioavailability compared to an FeSO₄ supplemented diet and replenished liver Fe and hemoglobin concentrations (Murray-Kolb et al., 2002).

Two other animal models which have been utilized to study biofortification efficacy are the chicken and piglet. Chickens are useful for nutritional studies as their results correlate well with human cell lines, and they respond quickly to malnutrition, especially low Fe (Tako et al., 2010). For the chickens, diet preparation consists of replacing all or some of their standard diet with control or modified plant material (corn, bean or rice) (Tako et al., 2011; Tako et al., 2013). Iron hemoglobin levels were higher in chickens fed diets containing high Fe beans in their diet, compared to the low Fe bean diet (Tako et al., 2011). In chicks fed high Fe corn, for a 6-week period, liver ferritin was higher indicating Fe absorption from the diet, which was composed of corn bred to contain more Fe (corn was 75% of the chick diet) (Tako et al., 2013). Chicks have also been used to assess the decreased mineral inhibition of diets containing grain with low phytate levels (Jang et al., 2003; Li et al., 2000). In both of these studies the total bone ash levels are higher in the chicks fed diet containing low phytate constituents (Jang et al., 2003; Li et al., 2000). Like the long term feeding studies in chicks, one 5-week study in piglets investigated the increased Fe absorption from beans in 28-day-old piglets (Tako et al., 2009). The piglets were fed with a diet partially containing either biofortified beans high in Fe or standard beans and weekly Fe hemoglobin

levels were measured. The biofortified bean containing diet increase the hemoglobin Fe, by approximately 18%, in the piglets; proving higher Fe in the fortified bean results in increased absorbed Fe (Tako et al., 2009).

Humans

The most accurate model to use to study bioavailability is people. In the case of low-phytate crops, numerous studies have analyzed their effect on Zn and Ca absorption in children and adults. Utilizing dual isotope ratio techniques, Mazariegos and colleagues found that low-phytate corn fed to Guatemalan school children for 10 weeks did not increase Zn absorption (Mazariegos et al., 2006). Also zinc supplementation combined with low-phytate corn is ineffective in improving growth velocity of 6 to 12 month infants suggesting no increase in zinc absorption in this infant population (Mazariegos et al., 2010). However, in adults consuming meals prepared from stable, extrinsically labeled corn, fractional Zn absorption increased as phytate concentration in corn is reduced (Adams et al., 2002; Hambidge et al., 2004). These Zn studies show a reduction in the antinutrient phytate improves Zn absorption in adults but studies in children were not as beneficial. Low-phytate corn fed to women increases fractional Ca absorption by 30% compared to meals containing corn without a reduced phytate concentration (Hambidge et al., 2005). However, stable, extrinsic Fe isotope labeled beans, bred to contain higher Fe, fed to Rwandese woman shows little nutritional improvement in Fe absorption. This is mainly due to beans having a rich concentration of phytic acid, impairing the Fe biofortification (Petry et al., 2012). Two studies using stable Fe and Zn isotopes to extrinsically label pearl millet show that varieties selected with higher plant Fe and Zn absorption improve Fe and Zn absorption in young Beninese women (Cercamondi et al., 2013) and young Belgian children (Kodkany et al., 2013). The conclusions from these studies show that while human testing is vital it is not without issues. The differing results seen between these studies underscore the importance that humans have regional, developmental and dietary differences which can impact these efficacy studies.

Carrots, genetically modified to uptake higher Ca, intrinsically labeled with a stable Ca isotope and fed to 30 healthy adults produce a 41% increase Ca absorption (Morris et al., 2008). Golden Rice intrinsically labeled with heavy water (deuterium oxide – ^2H - β -carotene) allowed researchers to measure the amount of absorbed β -carotene in 5 adults, proving that genetically modified rice contains nutritionally beneficial vitamin A precursors (Tang et al., 2009). Similar studies with intrinsically labeled Golden Rice fed to children result in an approximately 60% absorption of recommended daily intake of vitamin A from a single bowl of rice (Tang et al., 2012).

The wide variety of model systems used to test fortification efficiency has bolstered the sciences of fortification by allowing researchers to quantify nutritional bioavailability, as well as test safety issues.

Golden rice: a tale of controversies and bureaucratic inefficiencies

The draconian rules and regulations established for the handling and use of GM plants are so demanding that it takes a minimum of ten years to prepare for (Potrykus, 2010a) and assemble all the data required for a regulatory dossier, not to mention the exorbitant costs involved (Perez-Massot et al., 2013). Let us examine the various obstacles and solutions that have been encountered during the development of Golden Rice.

Intellectual property (IP) rights were the first hurdles that were encountered. Academic scientists are normally free to exploit the public knowledge provided by patented inventions. However, this all changes when the product they develop begins being developed for distribution (Potrykus, 2010a). During the development of Golden Rice the scientists had no idea that they were potentially infringing on 70 patents belonging to 32 patent holders. This problem arose quickly but was solved relatively rapidly through a compelling compromise involving all the patent holders and the developers (Enserink, 2008). They all made some sort of financial or personal sacrifice coming to view this creation as a humanitarian project whose long-term viability was important for hundreds of millions of people in developing countries.

Lack of financial support from the public domain was the next hurdle. Universities fund ideas not the development of products. The creators of Golden Rice were forced to work with a company to commercialize the product. The scientists made sure that the technology remained readily available for 'humanitarian use' in developing parts of the world. Unfortunately, the companies involved could not continue this collaboration for long, as the trouble mounted (see below) the chance for a financial return at the level of the investment was too low. Financial support is now been received from philanthropic and other visionary organizations such as The Rockefeller Foundation, USAID, and the Syngenta Foundation (Enserink, 2008).

The most dramatic bottleneck has been GM-regulation. A decade of annoyance is difficult to quickly summarize but let us take an overview of the events. Deletion of selectable markers in Golden Rice took two years (Potrykus, 2010a). Regulatory authorities prefer that antibiotic selectable marker genes be deleted. This was done despite the fact that there is no convincing data documenting that the antibiotic markers used with the rice could have any adverse effects on the consumer or the environment. Furthermore, regulatory authorities do not accept complicated integration patterns of the 'transgenes'. Golden Rice requires the use of several genes to produce the desired phenotype, thus regulatory 'safeguards' deem this procedure dangerous (Potrykus, 2010a). Meanwhile

standard breeding processes lead to uncontrolled integration and go unregulated. A complicated gauntlet of bioengineering safeguards requires many genetic manipulations and is required prior to commercialization of GM crops. This multiyear project was spearheaded by Syngenta who then donated the rice lines to the multinational effort (Potrykus, 2010a). Golden Rice also requires an international breeding program. Rice breeders need to develop locally optimized Golden Rice using the most popular varieties in their native countries (Coghlan, 2013). Sadly, regulators make exchange of seed so complicated that it took more than two years to transfer samples between countries. Regulatory agencies also require 18 months of greenhouse type experiments prior to any field testing (Potrykus, 2010a). This despite the fact that no scenario has been envisioned for any environmental risk posed by the use of Golden Rice (Perez-Massot et al., 2013; Potrykus, 2010a, b). With these restrictions, preparing and characterizing a single transgenic line of Golden Rice becomes demanding and expensive. This creates a situation where it is impossible to try and deregulate several independent transgenic events. Furthermore, this makes for a perilous situation to put all your resources into a single line without being able to screen the varieties in small field tests. Even after all these arduous events have been documented the work STILL has to be passed through a further series of bureaucratic channels.

These problems are all BEFORE we consider the central theme of this review-nutritional testing. The results of a Golden Rice feeding trial, funded by the U.S. National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and the U.S. Department of Agriculture (USDA), were published online in 2009 to little notice by *The American Journal of Clinical Nutrition* (Hvistendahl and Enserink, 2012; Tang et al., 2009). The researchers show that a single bowl of this rice can supply half of some people's vitamin A requirement — the most convincing evidence so far that golden rice can, in fact, be a useful tool in fighting malnutrition. However, within 30 days, Greenpeace China claimed that the study had violated a Chinese government "decision to abort plans for the trial," which it called "a scandal of international proportions." The group did not offer evidence to support its allegations, but Tufts University (where a majority of the work was conducted) said it was "deeply concerned" and are conducting a review (Hvistendahl and Enserink, 2012). Subsequently, the Chinese government has punished several China-based researchers who were involved in the feeding study, removing them from their jobs. According to the government, the researchers did not obtain proper approvals before carrying out the study. Tufts, after spending more than a year carrying out its own review, has also decided the work was not conducted in full compliance with their policies or federal regulations. They concluded that the researchers did not adequately explain the nature

of golden rice to study participants and made some changes in the study without getting approval from internal sources that are meant to review all research involving human subjects. The first author on the work has now been banned from conducting research on human subjects for two years. For two years after that, any human subject research that the investigator conducts will be supervised.

At this point the promise of Golden Rice remains while the difficulties persist. It is readily apparent that the scientific participants have been earnest and diligent, but also optimistically naive (Enserink, 2008; Hvistendahl and Enserink, 2012; Potrykus, 2010a, b). Genetic modification regulations have delayed the deployment of golden rice and still could shelf the technology. After several decades, there is no scientific justification for present GM-regulations (Miller, 2009). In fact, given that no harm has yet arisen from the use of this technology it is time to change the current regulations, which are based on precautionary principles, and transform our guidelines to science-based regulations based on the engineered product rather than the process used to produce the given trait (Chassy, 2010; Potrykus, 2010b).

CONCLUSIONS

Fortification of foods remains a cost effective strategy to enhance nutrition in under developed and nutrient starved regions where processed fortification would be too costly to sell and too rural to adequately supply (Chassy, 2010; Gomez-Galera et al., 2010; Potrykus, 2010b; Rommens et al., 2004). Specifically, genetic modifications offer the most promise for the future of biofortification (Gomez-Galera et al., 2010) but also produces the biggest hurdles in accomplishing the goal of malnutrition eradication (Gartland et al., 2013; Weale, 2010). Despite all the plant level biofortification efforts, few studies have been conducted to prove, ultimately, that the plants have more bioavailable nutrition. Utilizing model systems, such as rodents and cell lines, provide researchers an avenue to test biofortification efforts prior to testing in humans. Despite little evidence that GM foods are deleterious to human health (Lack, 2002; Mayer et al., 2008; Potrykus, 2010b) it will take time to get these fortified plants through the bureaucratic cycle and into the fields, all the while those who most need these improved foods will continue to be undernourished.

In the meantime, there are numerous methods which can help us to further advance biofortification efforts. For instance, introduction of a β -carotene rich orange sweet potato into Ugandan households decreased vitamin A inadequacy in children and women (Hotz et al., 2012). Utilizing techniques which modify the plant's own DNA — not incorporation of a transgene(s) from another entity, new enhanced varieties can be created (Shukla et al., 2009) without negative public perception and hurdles that other GM plants struggle to clear (Cressey, 2013). Lastly,

researchers have invented an artificial gut with the use of novel 3D hydrogel scaffold covered with Caco-2 cells (Shah et al., 2006; Sung et al., 2011). This new *in vitro* model could help bridge the gap from animal studies to humans and possibly lessen any perceived concerns of GM plants. In the end, the studies involving biofortification of crops need to show added value and those utilizing GM also need to continue to show they are safe for human consumption to better combat global malnutrition.

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